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U.S. DAIRY FORAGE RESEARCH CENTER

1992 RESEARCH SUMMARIES

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Agricultural
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United States
Department
of Agriculture

March 1993

U.S. DAIRY FORAGE RESEARCH CENTER, USDA-ARS
Madison, WI 53706

Dear Reader:

Larry D. Satter

It is a pleasure to update our progress by bringing you these summaries of recent research. The U.S. Dairy Forage Research Center is a unique part of the national research program of the Agricultural Research Service, U.S. Department of Agriculture. The Center's mission is to build a knowledge and technology base for the dairy industry to fully exploit the use of forages in the production of milk. The Center has agricultural engineers, plant and soil scientists, microbiologists, ruminant nutritionists and a chemist working together to increase the efficiency of forage production and utilization by dairy farmers. We function in close cooperation with the Agricultural Experiment Stations of several states. The Center is located on the campus of the University of Wisconsin, Madison, and has "Cluster" locations in St. Paul, MN, Ames, IA, East Lansing, MI, and Ithaca, NY. The Center's research farm with facilities for 300 milking cows is located on 63 acres of USDA land on the banks of the Wisconsin River in Prairie du Sac, WI. An additional 1200 acres of adjacent land is utilized by the Center by agreement with the U.S. Department of the Army. The Center was established in 1980 and has made steady growth since. At present there are fifteen scientists: ten at Madison, and one at each of three Cluster locations, and two at the St. Paul, Minnesota Cluster location. Scientists hold faculty appointments in university departments and provide supervision for approximately 15-20 graduate students and 5-10 post doctoral fellows.

The three work groups around which roughly two-thirds of our activity is centered have been functioning well. The cell wall work group has been together longer than the other two groups so they have a more highly integrated or multidisciplinary approach to experimentation. Publication of the proceedings of the symposium sponsored last year by the cell wall work group is expected soon.

The protein work group includes several University of Wisconsin faculty and has been meeting monthly. This group will sponsor a half-day workshop at our annual meeting in March. The systems work group met in East Lansing in October and again in January in Madison. Our effort to strengthen the 'systems' program by hiring a plant scientist with extensive experience with crop growth models was delayed, but we are on track again and hope to have a person on board shortly.

You will note that we have added a new section to our report this year. We wish to highlight in this report the progress the cell wall work group has made during the past five years and to indicate where the group is headed in the future. We are experimenting with the color section towards the front of the report to convey this message. Our intent is to give some perspective to the research we are doing and to give the reader a feel of how research progress is much like the unfolding of a story. It is hard to portray that in short annual accounts of progress. We hope to have a 'story' section each year, highlighting progress over time of our research program.

We are pleased and very proud of the way Center scientists from diverse disciplines interact and bring their collective insights to bear on the problems of forage production and utilization. This collection of research summaries illustrates the progress they are making in developing information to help dairy farmers utilize their resources more effectively. The research is intended to benefit producers of forage crops, dairy farmers and the consumers of dairy products.

Sincerely,

Larry D. Satter

Larry D. Satter, Director
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Acknowledgment

Appreciation is expressed to Gloria Palmer for her interest in and dedication to the task of typing and assembling this annual research summary.

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PUBLICATIONS

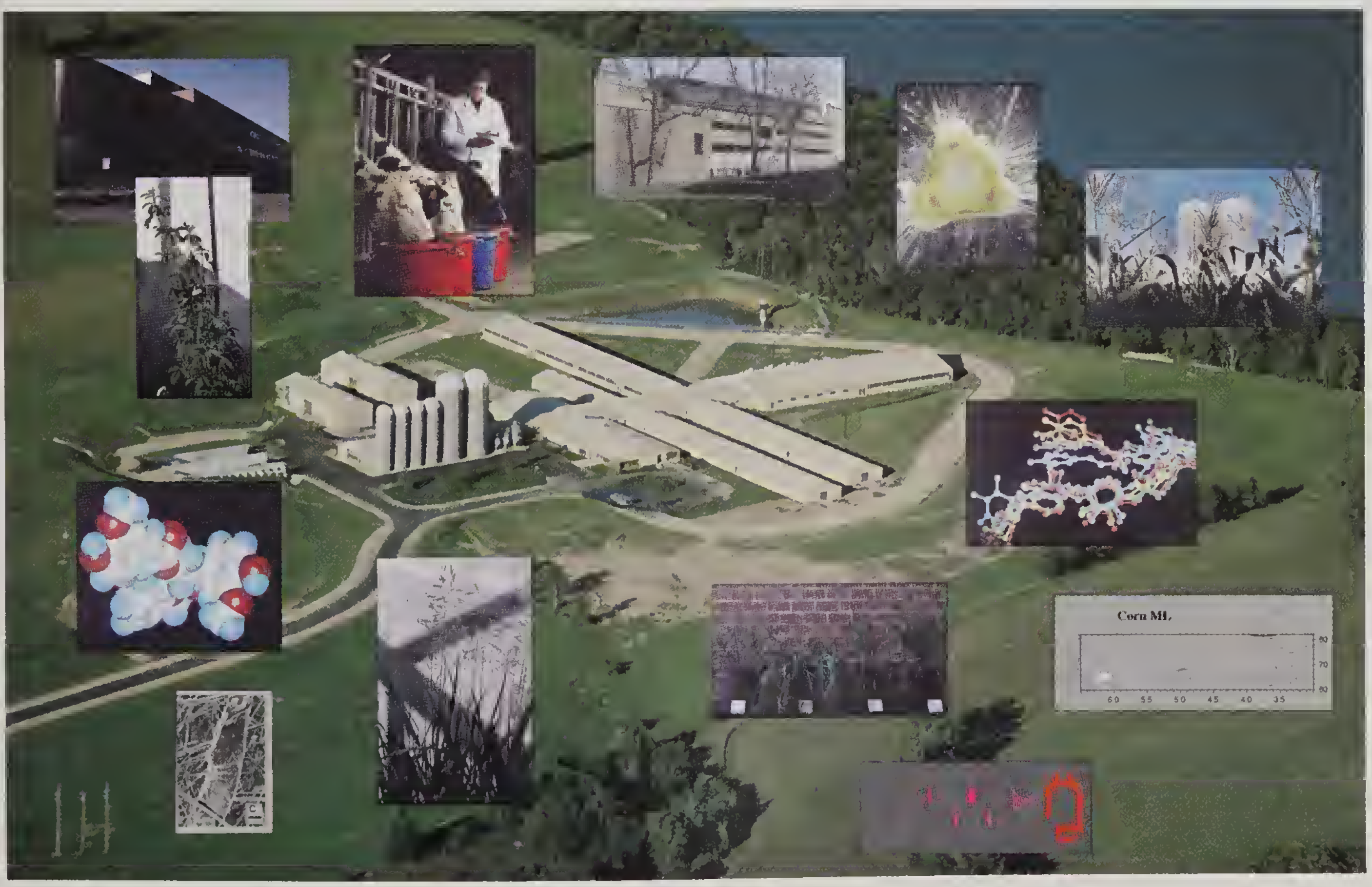
THE CELL WALL CHARACTERIZATION AND UTILIZATION WORK GROUP

Plant cell wall composition and organization, in particular cross-linkages among wall components, critically impede digestion and utilization of forages. Scientists at the USDFRC, realizing that a multidisciplinary approach is needed to investigate cell wall degradability, formed the Cell Wall Characterization and Utilization work group to define and study specific chemical and physical characteristics of plant walls that limit forage utilization. The work group consists of six scientists, each representing a specialized field and bringing unique skills to solve these problems. This team effort provides a forum for exchanging expertise, standardizing methodology and terminology, and integrating individual research findings into a broader perspective and direction.

Organization of the group is informal, without a designated leader, and emphasizes intensive discussions of issues related to forage walls and their utilization. Lengthy and lively discussions during the regular meetings of the group have resulted in numerous collaborations. Currently there are ten active cooperative projects including: evaluation of inbred corn lines for wall composition and digestion kinetics, identification of a cicer milkvetch inhibitor of cellulose degradation, and elucidation of the molecular structure of lignin-polysaccharide linkages. The team's collaborative research has produced numerous co-authored papers on topics ranging from regiochemistry of *p*-coumaric acid esters in corn lignin to bias in the data on cell-wall composition estimated by NIRS.

The group recently organized an international scientific meeting. The International Symposium on Forage Cell Wall Structure and Digestibility was held in Madison, WI in October of 1991. This meeting brought together leading researchers on cell-wall structure and digestion for intensive discussions and resulted in the publication of a reference book which will be available in April 1993. One hundred sixty-five scientists from fifteen countries attended the four day symposium where twenty invited speakers presented plenary papers and participants took part in evening poster sessions.

The existence of this multidisciplinary group has allowed its members to attack problems in a comprehensive manner and has significantly increased progress toward the goal of understanding, and ultimately improving, cell-wall digestibility. We look forward to an exciting and fruitful future as a multidisciplinary team focused on the complexities of the plant cell wall.



Dwayne Buxton (Fig. 1), a plant physiologist/agronomist with the USDA-ARS Field Crops Research Unit at Ames, Iowa, is involved with plant growth and development studies aimed at determining whole plant relationships associated with improved cell wall digestibility. Recent studies have focused on defining the temporal sequence in which key lignin synthesizing enzymes are active in producing lignin precursors and lignin in plant tissues. Studies with corn internodes (Fig. 2) have allowed precise definition of when this occurs in relation to plant development. In grasses such as corn, lignin accumulates first in the upper, then the middle, and lastly in lower internode regions.

Fig. 1. Dwayne Buxton and Teresa Morrison (a research associate), measuring internode development of corn plants.

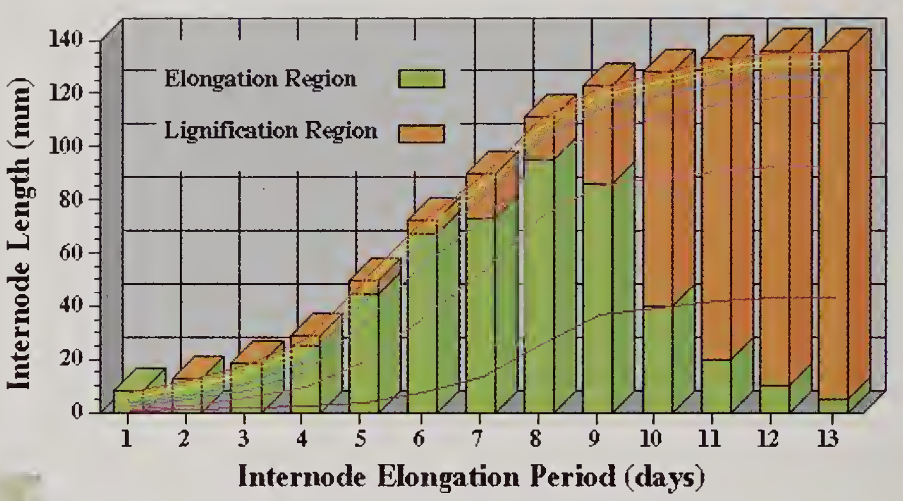


Fig. 2. Corn stem internode development vs time. Lines reflect elongation of internode regions over a 13 day period. By day 2 the top 2 mm stopped elongating and began lignifying. During extension of a grass internode, the lower portion is more digestible than the upper portion. Along the stem, bottom internodes are more mature and more highly lignified than top internodes. Because more than one internode is elongating at a time during stem growth, there is a complex pattern of young and old tissues along grass stems.

Fig. 3. Hans Jung running HPLC analysis of phenolic acids.

Hans Jung (Fig. 3) is a dairy scientist with the USDA-ARS Plant Science Research Unit at St. Paul, MN and is a cluster scientist of the USDFRC. His research project is concerned primarily with the identification of cell-wall components that limit forage digestion by dairy cattle. Special emphasis is given to the possible role of ferulic acid as a cross-linking molecule between arabinoxylans and lignin in grasses and its effect on ruminal digestibility of cell walls. Current research is directed toward determination of the genetic variability for lignification and cell-wall digestibility in alfalfa, smooth bromegrass and corn. Unique forage germplasm identified in these studies will be used to test the effect of cell-wall organization on animal performance.

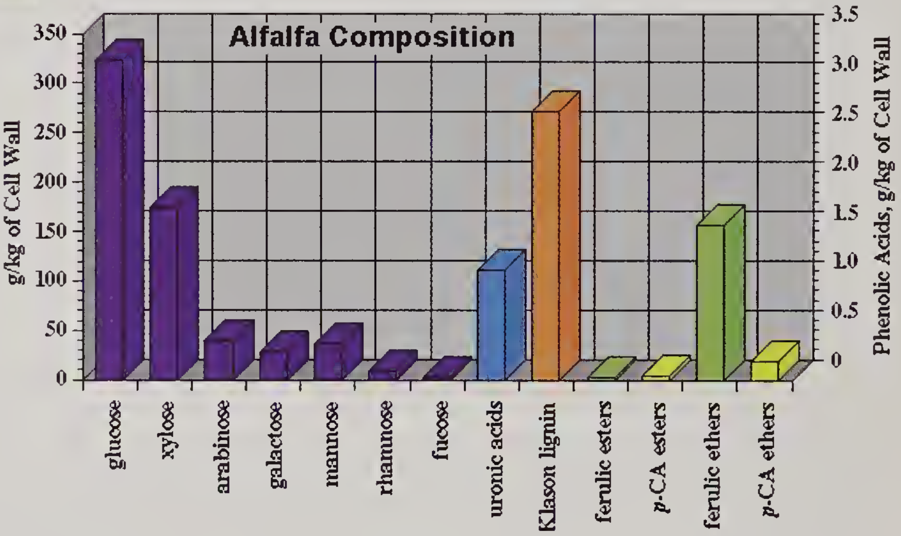


Fig. 4. Cell wall composition of stems from alfalfa selected by R. R. Smith for low in vitro dry matter digestibility and high acid detergent lignin. Despite the low absolute concentrations of phenolic acids (shown expanded, right axis) these structures may be critical to wall digestibility.

Fig. 5. Ron Hatfield purifying an enzyme by low-pressure liquid chromatography at 5 °C.

Ron Hatfield (Fig. 5) is a plant physiologist dealing with wall matrix interactions. He is primarily interested in biochemical mechanisms that lead to the formation of cross-linkages among wall components, their developmental regulation, and determination of their structural and functional roles within plants.

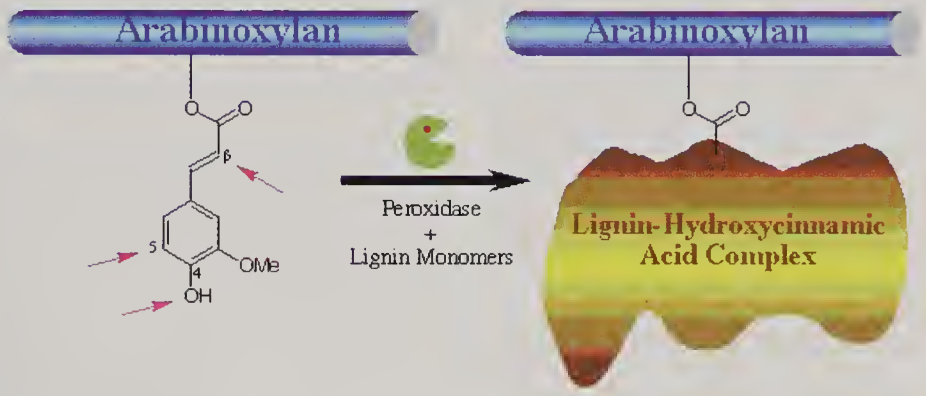


Fig. 6. Active co-polymerization of feruloylated arabinoxylans into lignins produces important cell wall cross-links important to the structural integrity of the wall.

A significant portion of the group's efforts has been directed towards understanding the role of hydroxycinnamic acids in cross-linking polysaccharides (particularly arabinoxylans) to lignins (Fig. 6). We have been the first to examine the co-polymerization of hydroxycinnamoyl esters into lignin and explore the complex array of structures that result. Current efforts involve searching for like structures in plant walls to determine pathways of incorporation and their biochemical implications. A recent study has determined the regiochemistry of *p*-coumaric acid attached to corn lignin (Fig. 7).

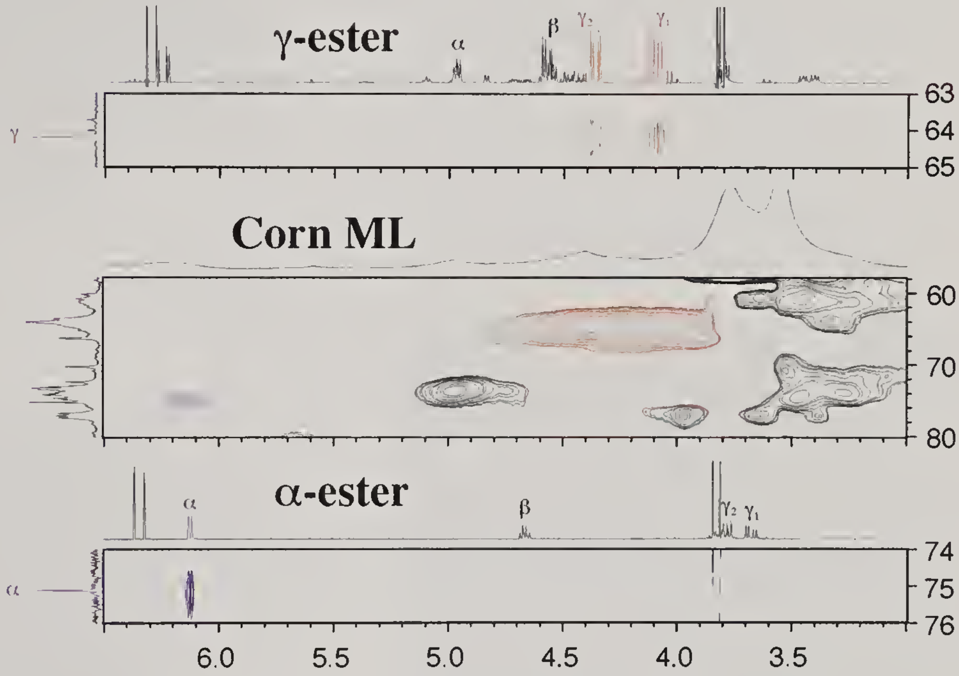


Fig. 7. Proton-detected 2D short-range ^{13}C–^{1}H correlation (HMQC) NMR spectra of corn and model compounds showing that p-coumaric acid in corn is attached exclusively at the γ-position. The biochemical implication is that preformed coniferyl p-coumarate dimers are involved in lignification and stresses the role of p-coumaroyl transferases that have, to date, not been identified.

Fig. 8. John Ralph and Richard Helm running 2D NMR spectra.

John Ralph (Fig. 8) is an organic chemist and NMR spectroscopist specializing in model compound syntheses and monolignol polymerization reactions. He is principally involved in studies aimed at detailing the mechanisms of incorporation of hydroxycinnamic acids into lignin-polysaccharide complexes. Acquisition of an NMR spectrometer by the Center has allowed the implementation of novel pulse experiments for the characterization of cell wall structures, including unambiguous regiochemical determinations (Fig. 7).

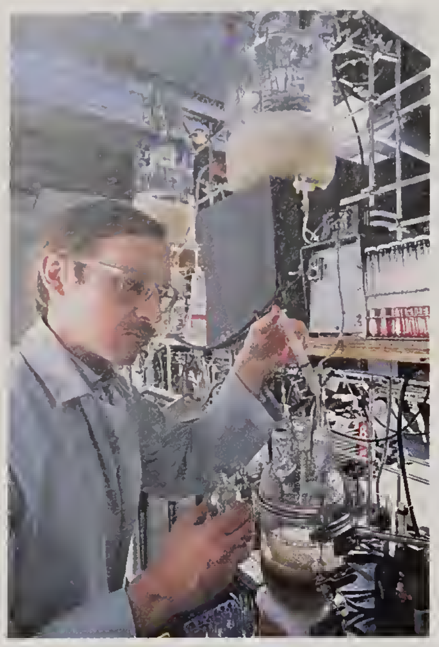


Fig. 9. Paul Weimer, with continuous culture system.

Fig. 10. Chris Odt at the scanning electron microscope (SEM).

Paul Weimer (Fig. 9), a microbiologist, applies his background in anaerobic biomass conversions to the study of ruminal polysaccharide fermentation. His primary interests have involved the role of substrate fine structural features on digestion kinetics, and the quantitative determination of fundamental fermentation parameters for fibrolytic bacteria. The latter studies have employed specialized culture devices (Fig. 9) designed for continuous, anaerobic delivery of insoluble plant polysaccharides. Coupling of these investigations to more traditional biochemical and microscopic (Fig. 10) studies has elucidated some of the factors controlling the formation of different fermentation end products by individual species of cellulolytic bacteria (Fig. 11). Current studies are aimed primarily at identifying the physiological factors determining the outcome of competition among individual fibrolytic species. Weimer's most recent work involves determination of the role of interactions among polysaccharides as a secondary determinant of digestion rate.

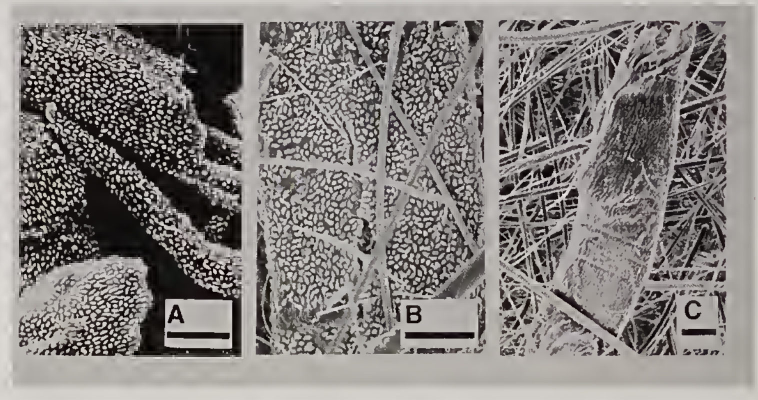


Fig. 11. A soluble agent in cicer milkvetch dramatically inhibits cellulose digestion. SEM has revealed that increasing concentrations of this agent result in successively greater detachment of fibrolytic bacteria from cellulose fibers. A. without, B. intermediate levels and C. maximal levels for inhibition.

Fig. 12. Dave Mertens measuring NDF.

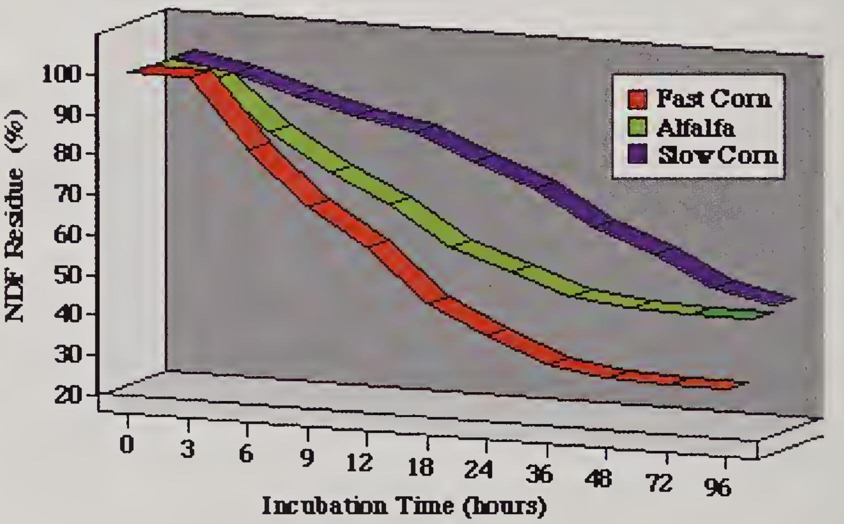


Fig. 13. The rate and extent of NDF-digestion from different plants vary greatly. Quantifying differences in digestion kinetics and identifying the chemical and physical characteristics that lead to these differences will allow the development of ways to improve forage digestion and utilization. Fast/Slow refers to digestion rates.

Dave Mertens (Fig. 12), an animal nutritionist, helps keep the group focused on the performance of animals on high cell wall diets as a critical issue for research. Because cell walls are bulky and difficult to digest, they often limit intake and digestibility, the primary areas of his research. Mertens has been developing a system for maximizing forage use in the rations of dairy cows by optimizing the NDF content of the ration. Once optimal NDF content of the diet is obtained, the most critical issue is maximizing utilization of cell wall consumed by the animal. To identify limitations in cell wall digestion, Mertens is investigating their digestion kinetics (rate and extent of digestion, Fig. 13).

ALFALFA ROOT ELONGATION CAN BE MONITORED WITH THE HERBICIDE FLURIDONE

L.L MEYERS, M.P. RUSSELLE, J.F.S. LAMB and D.K. BARNES

Michael P. Russelle

Introduction

Maintaining excellent quality in ground water supplies is a high priority from the perspective of human and livestock health. Avoiding ground water contamination likely will be less expensive than removing substances from contaminated water supplies. Nitrate is one of the compounds that has been found in many rural wells and ground water aquifers. Nitrate is a natural result of soil organic matter decomposition, but is also formed in soil after application of nitrogen fertilizer, animal manures, municipal waste, and compost. Nitrate is an important source of nitrogen for plants and is readily absorbed by roots during plant growth. But excess nitrate moves through the soil with water. If nitrate moves below the root zone of annual crops like corn and soybeans, it will likely continue downward until it reaches ground water.

Deeply rooted perennial plants, like alfalfa, have the potential to be scavengers of nitrate from deep in the soil. In order to optimize alfalfa's role in removing nitrate before it reaches ground water, it would be desirable to develop varieties with very rapid root elongation rates. There have been no affordable methods that allow selection of plants with fast growing roots.

However, a herbicide called fluridone attracted our interest because it is long lived in soil, it does not move in soil, and it produces nonlethal, distinctive visual symptoms in plant leaves. Fluridone causes bleaching of new leaves in most plants, including alfalfa. It interferes with carotene synthesis, so new tissues are white. Because new growth usually occurs near the top of alfalfa stems, it is easy to identify plants that have absorbed the herbicide. If this material could be applied at a specified depth, it might last long enough and stay in place so that we could easily select plants that showed the symptoms. However, for plant breeding purposes, the plants would need to recover from fluridone effects.

Our objectives were: (1) to evaluate the feasibility of using the herbicide fluridone to indicate rate of root elongation of alfalfa; and (2) to evaluate differences in root elongation rates within and among different alfalfa populations.

Materials and Methods

A series of experiments was conducted to determine: (1) the rate of fluridone to use; (2) the ability of alfalfa to recover from fluridone symptoms after transplanting; (3) the potential for fluridone release from alfalfa roots once the top portion has been transplanted; (4) the sensitivity of different alfalfa varieties to fluridone; and (5) the variability in root elongation rate within and among different alfalfa plant populations. All experiments were conducted in the greenhouse in a soil-sand mixture.

Rates of fluridone from 2 to 50 mg/kg were added to a layer of soil at the bottom of 15-cm diameter pots and covered with untreated soil in the first experiment. Alfalfa seedlings were transplanted to each pot (4 replicates per treatment) and were observed for symptom development. After most plants showed symptoms, plants from 3 replicates of the 25 and 50 mg/kg rates were transplanted to a sand bench, and were observed during their recovery from fluridone toxicity. Roots from plants in the fourth replicate were removed from the soil, cut into small segments, and added to untreated soil in new pots. Alfalfa seedlings were transplanted into these pots and observed for fluridone symptoms. Twelve alfalfa varieties were seeded in 15-cm diameter pots filled with untreated soil, were treated with 25 mg/kg fluridone after 42 days of growth, and were observed for symptom development. The alfalfas varied in fall dormancy, root morphology, and nodulation effectiveness. In the final experiment, a layer of fluridone-treated soil was placed at the bottom of a large pit and was covered with 80 cm of untreated soil. The same 12 alfalfa varieties were seeded in 3 replicates and the development of fluridone symptoms was monitored.

Results and Discussion

The best rate of fluridone application was 25 mg/kg. Symptoms developed rapidly at this rate, and there was no apparent change in plant growth rate. Only a few plants died after transplanting, and those had been removed from the treated pots several days after symptoms developed. All other transplants recovered from the fluridone and regrew normally. No symptoms were observed in pots containing root pieces from fluridone-treated plants. Therefore, it is unlikely that plants would show symptoms in the field after absorbing fluridone from the decomposing roots of their neighbors. All plants from the various alfalfa populations showed symptoms within 6 days of fluridone application to the pots, so we conclude that most alfalfas could be expected to be uniformly susceptible to fluridone at the 25 mg/kg rate.

The final experiment showed that there were large differences in root elongation rate within alfalfa populations, but little difference between the averages of each population. Root elongation rate varied from an average of 1.52 cm/day for the fastest growing individual plants in each population to an average of 1.16 cm/day for the fastest 80% of each population. The fastest populations tended to be strongly taprooted types. Those that have large numbers of lateral and fibrous roots tended to have slower root elongation rates. There was no effect of fall dormancy or of nodulation effectiveness.

Conclusion

Fluridone appears to be an excellent tool for evaluating root elongation rate. Because plants can recover from its effects, fluridone will also allow plant breeders to select plants for use in developing new varieties. We found that sufficient variability exists both among and within alfalfa varieties to provide the opportunity for selecting alfalfa for rapid root elongation rate. We will be evaluating alfalfa root growth rate to 180 cm in the field in 1993 using this new technique, with the goal of developing new alfalfa germplasms for use in removing nitrate from contaminated subsoil.

ROOT PRODUCTION AND LOSS RATES IN FORAGE LEGUMES

M. DUBACH and M.P. RUSSELLE

Introduction

Plant roots are a major source of organic matter in the soil. In addition, forage legumes can enhance soil fertility through their ability to fix nitrogen (N) from the atmosphere. Annual input of fixed N to the soil under continuous alfalfa is estimated to be more than 100 kg/ha. In newly established stands of alfalfa and birdsfoot trefoil with grass, fixed N transfer of up to 20 kg/ha has been measured the first season in the grass herbage. It would be desirable to manage this N input; for example, in some eroded soils, we may wish to increase the N content, whereas in others we may need to limit N inputs because of nitrate leaching problems. Legumes with different capacities to add N to the soil are needed in these cases.

But we do not know how this fixed N is released into the soil. Root decomposition is a possible pathway of N loss from plants but is very difficult to quantify under field conditions. The recent development of minirhizotrons, which are a combination of a miniature video camera and clear plastic tubes that are inserted in soil, allows us to observe the same roots many times during the season. If video pictures from different dates are compared, root production and loss can be measured. Our objective was to quantify root production and loss rates in seedling stands of alfalfa and birdsfoot trefoil under field conditions.

Materials and Methods

The experiment was conducted on a silt loam soil at the University of Minnesota Rosemount Agricultural Experiment Station near Rosemount, MN. Four pits (3 X 2.5 X 1.5 m deep) were dug, walls were reinforced with plywood, and each pit was covered with a roof. Opposite sides of each pit were considered replicate plots, thus establishing a total of 7 replicates. Minirhizotron tubes were inserted horizontally in each plot at midline depths of 7, 12, 20, 30, 40, and 70 cm. Tubes were offset by 15 cm so that each would be located under a different plant row. Plots were amended with P, K, and lime according to Univ. of Minnesota soil test recommendations. Saranac alfalfa and Norcen birdsfoot trefoil were seeded in mid May 1991 in rows spaced 15 cm apart and oriented directly over the observation tubes. Each species was seeded in a sub plot within each replicate. Seeds were inoculated with appropriate rhizobium. Stands were thinned to about 200 plants/m^2, resulting in a 30-mm spacing between plants within rows.

Beginning 6 June 1991, 40 images (13 X 17 mm) were taken on each side of the tubes for each species, resulting in 80 images per species per date. Recordings were made weekly until 31 October 1991. Images were edited into time-lapse sequences using conventional editing techniques. We were not able to see when a root died but could identify roots that decomposed. Dates of appearance and disappearance of every root were entered on a spreadsheet, which calculated average root lifetime and total numbers of roots produced and lost. Total root count for a species from each tube was considered to be one observation unit.

After detailed analysis to assure that neither accuracy nor precision of data was compromised, we used fewer images at some depths, i.e., the time-lapse sequences were comprised of every other image along the tube and two-week intervals. Data were analyzed as a strip-strip plot design, with one strip being plant species and the other being depth. For brevity, data reported here are for fine, nonsecondarily thickened roots only.

Results and Discussion

Herbage was harvested on 24 July and 30 August (about 10 and 15 weeks after planting); total yields were 0.44 kg/m^2 for alfalfa and 0.57 kg/m^2 for birdsfoot trefoil. Total numbers of roots produced over the season by alfalfa ranged from 602 at 7 cm to 188 at 70 cm, and by birdsfoot trefoil ranged from 696 at 7 cm to 242 at 70 cm. Numbers of disappeared roots in alfalfa ranged from 336 at 12 cm to 49 at 70 cm and in birdsfoot trefoil ranged from 334 at 7 cm to 43 at 70 cm. Thus, 64% of all roots died and decomposed at the 12-cm depth in alfalfa, in contrast to only 34% in birdsfoot trefoil at that depth. At the 70-cm depth, 28% of all alfalfa roots and 18% of birdsfoot trefoil roots died and decomposed. Using these values and root length densities measured in separate core samples, we estimate that each species lost about 30 km of roots per square meter (76,000 miles per acre) within the first growing season. This loss of roots occurred without any measureable stand decline. An example of the time course of observed vs. decomposed roots is shown in Figure 1. Although root turnover will vary with both biotic and abiotic stress (drought, insect and disease incidence, etc.), these values serve to emphasize the tremendous level of below ground activity in plant biomass accumulation and loss. In addition, they demonstrate that root data obtained from soil cores taken at only one time during the season can give a strongly biased view of plant rooting behavior.

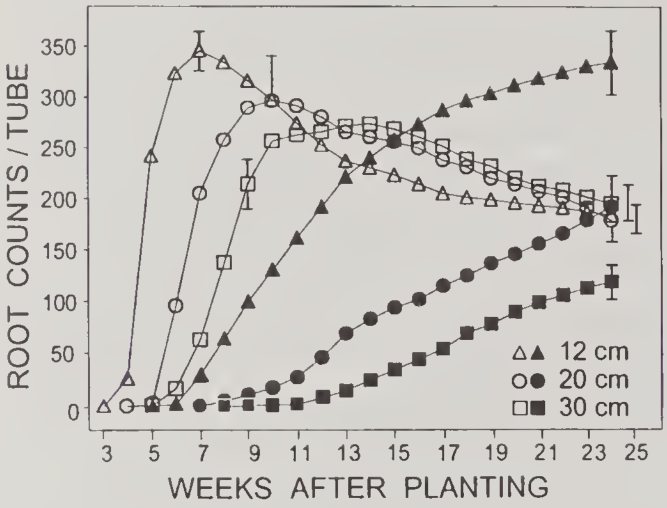


Figure 1. Total visible roots (open symbols) and total disappeared roots (closed symbols) of alfalfa at 3 depths in the soil. Counts are for 52 cm of tube length and are means of 7 replicates.

Conclusion

The minirhizotron system was an excellent tool for studying root production and disappearance. Because of the variability inherent in root distribution and longevity, root studies require large numbers of observations. We collected 6720 images every week. An important outcome of this research was our analysis of the number and frequency of images needed to quantify root dynamics. We were able to reduce the cost of collecting this information by more than 67%. Thus, this research provides some of the first values of root turnover for these important forage legumes and suggests ways that others can achieve significant savings in the cost of conducting root turnover research.

EFFECT OF NATURAL SELECTION FOR PERSISTENCE ON RESPONSE TO *FUSARIUM OXYSPORUM* IN RED CLOVER

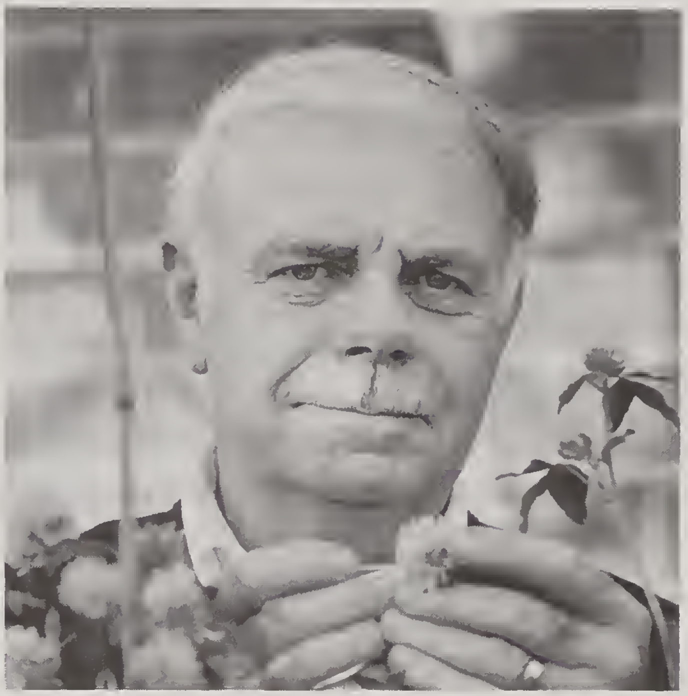
B.C. VENUTO, R.R. SMITH and C.R. GRAU

Introduction

Red clover (*Trifolium pratense*) is one of the leading forage legumes in the U.S., Canada, and northern and eastern Europe. However, each year diseases incited by fungi and viruses cause substantial losses in production and quality. The most practical means of controlling red clover diseases is to incorporate genes for disease resistance into agronomically acceptable cultivars or to develop new germplasm with resistance. Root rot complexes of forage legumes, especially the clovers, have been of concern to scientists and growers for many years. Organisms of the *Fusarium* species have been isolated most consistently from diseased roots. In Wisconsin, *Fusarium oxysporum* is the most prevalent *Fusarium* species isolated from red clover plants and from the soil. The objectives of the current research were to evaluate red clover cultivars and germplasm for their response to *F. oxysporum* and to relate this response to persistence of red clover.

Materials and Methods

Red clover germplasm developed in Wisconsin over the past four decades and a composite of the 1940's Wisconsin Common were included in this study. Cultivar Lakeland represented the germplasm developed in the 1950's, Arlington the 1960's, Marathon the 1970's, and population C11 the 1980's. For *F. oxysporum* evaluation, roots of 6-wk-old seedlings are trimmed to 3 cm length and immersed for 20 min in a potato dextrose broth containing 1 x 10^7 *F. oxysporum* microconidia per ml. Seedlings are then transplanted to sterile media of a soil:sand mixture (1:1 v/v). Seedlings are incubated for 8 wk and then evaluated. Roots of each seedling are evaluated according to a disease severity index (DSI) where 1 = a clean, white root with no visible sign of vascular browning to a 5 = death. A mixture of three *F. oxysporum* isolates retrieved from red clover plants grown in Ashland, WI, in 1988 was used (C.R. Grau, personal communication). Data on *F. oxysporum* response are the means of three separate evaluations and agronomic data are the means of two field tests conducted on the Agronomy Farm, Arlington, WI.



Richard R. Smith

Results and Discussion

A major emphasis of the red clover improvement project at the University of Wisconsin over the past five decades has been to increase persistence and forage production of the species. Routinely, surviving plants in three- and four-year-old stands are selected and incorporated into the adapted germplasm. Both forage yield and persistence have steadily improved (5.4 vs. 7.6 Mg ha^{-1} and 10 vs. 82% stand, respectively) over this period (Figure 1 and Table 1). Correspondingly, the level of resistance to *F. oxysporum* has improved (3.00 vs. 2.16 DSI) over this same period apparently as a result of natural and/or indirect selection. Whether the association between resistance and persistence is causal in nature or merely associative is not clear at this time; however, it will be more completely defined in ongoing and future research.

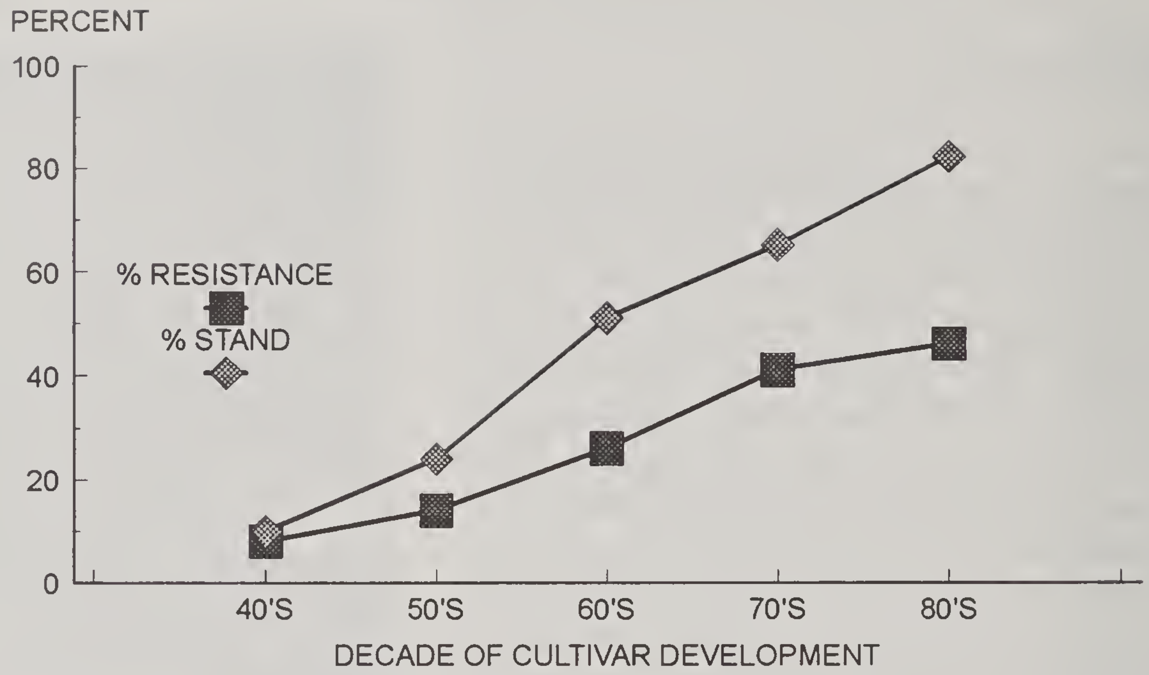


Figure 1. Percent stand and percent resistance to F. oxysporum relative to decade of cultivar/germplasm development.

Conclusions

Repeated selection and intercrossing persistent red clover plants from four-year-old stands have improved persistence of red clover germplasm. Correspondingly, resistance to the soil-borne fungus, *Fusarium oxysporum*, has increased which undoubtedly contributed to the improved persistence of the germplasm.

Table 1. Frequency distribution and mean disease severity index (DSI)+ of the response of red clover germplasm to *Fusarium oxysporum* and third year yield and percent stand at the end of three growing years in Wisconsin.

		Percent of plants with DSI of					Mean	DM	%
Germplasm	Decade	1	2	3	4	5	DSI	yld#	st
Common	40's	21	25	22	16	16	2.81	5.4	10
Lakeland	50's	14	25	22	25	14	3.00	6.3	24
Arlington	60's	26	23	18	18	15	2.73	6.7	51
Marathon	70's	41	24	15	11	9	2.23	7.6	65
C11	80's	40	25	20	9	6	2.16	7.6	82

+ DSI: 1 = no symptoms, resistant; 5 = dead plant, susceptible.

# Dry matter yield in Mg ha^{-1}.

PERFORMANCE OF RED CLOVER WITH AND WITHOUT COMPETITION OF GRASSES

R.R. SMITH and D.K. SHARPEE

Introduction

Most frequently red clover (*Trifolium pratense*) is grown in mixtures with forage grasses or other forage legumes. However, the development and testing of cultivars and germplasm are generally done in a monoculture environment under optimum conditions. The objective of this study was to assess the predictability of the performance of red clover cultivars and germplasm populations grown in pure stands (monoculture) with their performance when grown in competition with grasses.

Materials and Methods

Two similar experiments were established in 1987 on each of three research farms: Arlington, Prairie du Sac and Marshfield, WI. Red clover was seeded in 2 m rows 50 cm apart when seeded with grass and in 2 m rows 25 cm apart when seeded without grasses. A split-plot design was used with perennial ryegrass (*Lolium perenne*), timothy (*Phleum pratense*), or no grass as whole plots and red clover rows as subplots. Red clover rows were overseeded with ryegrass and timothy. The herbicide Poast (0.2 kg ha^{-1} a.i.) was applied to all plots in May 1990 to destroy all grass for final forage yield evaluation of the clover. Experiment one (Cultivar) evaluated 12 cultivars (Arlington, Atlas, Florex, Kenstar, Lakeland, Marathon, Persist, Prosper I, Reddy, Redland II, Ruby, and Starglo) and experiment two (Germplasm) evaluated 44 Wisconsin experimental populations and the two cultivars, Arlington and Marathon. Red clover performance was assessed as visual estimates of percent stand (percent of ground cover per 2 m row) 6 wk after establishment and in April of 1988, 1989, and 1990 and for residual forage yield in June 1990. All herbage was removed twice each year in early June and in late July (approximately 20% bloom).

Results and Discussion

Few differences were observed among cultivars or germplasm populations for percent stand whether with or without a companion grass until the end of the second year. By the beginning of the third year, significant differences among cultivars and among germplasm populations were evident both in pure stands and within companion grasses at all three locations (data not presented). The differences among cultivars became greater by the end of the third year and the beginning of the fourth; however, performance in pure stand was not predictive of the performance with either grass as evidenced by the poor correlations between pure stand and grass companion (Table 1). Differences among germplasm populations were greater than among cultivars. As a result, the performance of these populations in pure stands was more predictive of their performance with a grass companion (Table 1.— low, but significant correlations).

Conclusions

The performance of red clover germplasm evaluated in a monoculture environment was not consistently predictive of its performance when grown in competition with either timothy or perennial ryegrass. It would appear, however, that the initial evaluation of these red clover germplasm populations could be accomplished in monoculture environments. On the other hand, if the appropriate germplasm (cultivars, population, etc.) of the companion grass which will be used with the clover can be identified, it seems appropriate to evaluate the germplasm in competition with the grass from the start of the selection program. At the least, as superior populations are identified and as differences between populations become smaller, final evaluations should be with a companion species for which the final product is to be used.

Table 1. Correlation coefficients between pure stands and grass companion competition for percent stand and forage yield for red clover cultivars and germplasm.

	Percent stand						Yield	
	Pure stand vs.							
Exp/Loc	PR	TIM	PR	TIM	PR	TIM	PR	TIM
	Apr 88		Apr 89		Apr 90		June 90	
CULTIVARS+								
Arl	0.13	0.06	0.57*	0.23	0.24	0.18	—	—
Pds	0.21	-0.05	0.40	0.17	0.27	0.01	0.45	0.34
Msf	0.05	0.41	0.16	-0.11	0.46	0.51	0.35	0.50
Comb.	0.05	0.35	0.27	0.23	0.23	0.32	0.55	0.63*
GERMPLASM+								
Arl	-0.02	0.08	0.57**	0.58**	0.03	0.35**	—	—
Pds	0.14	0.16	0.63**	0.36**	0.38**	0.22	0.06	-0.13
Msf	0.21	0.09	0.45**	0.27*	0.59**	0.44**	0.12	0.23
Comb.	0.08	0.24	0.74**	0.67**	0.54**	0.47**	0.21	0.32*

+ Cultivars (n = 12); Germplasm (n = 50).
*,** Significant at 5 and 1%, respectively.
Abv.: PR = perennial ryegrass; TIM = timothy; Arl = Arlington; Pds = Prairie du Sac; Msf = Marshfield.

CHEMICAL REGULATION OF GROWTH, YIELD, AND DIGESTIBILITY OF ALFALFA AND SMOOTH BROMEGRASS

J.E. BIDLACK and D. R. BUXTON

Chemical regulators have the potential to increase yield and improve digestibility of plants. Recent research has revealed that commercial growth regulators can improve digestibility of grasses and manipulate the quality and yield of grass-legume mixtures. Investigations have generally shown that quality of grasses and grass-legume mixtures can be improved, but only at the risk of lowering yield. In this study, two classical plant hormones and one experimental chemical were used to study fundamental plant growth responses in forages and to determine whether or not forage quality can be manipulated without sacrificing yield. The hormones used were: gibberellic acid (GA3), which stimulates internode elongation; α-naphthalene acetic acid (NAA), an auxin that causes cell elongation; and o-benzylhydroxylamine (OBHA), a lignification inhibitor. The objectives of the study were to determine the effect of these growth regulators on forage growth and digestibility and to determine relationships between chemically altered growth and digestibility.

Materials and Methods

'Arrow' alfalfa and 'Barton' smooth bromegrass were established in 25-cm pots with a 3.8-L capacity in a greenhouse and harvested twice before regrowth was used for chemical treatment. Each pot contained either three alfalfa plants or several smooth bromegrass plants at the time of chemical application. Pots where placed adjacent to each other so that regrowth represented a growing sward. High pressure sodium lamps supplemented sunlight to provide a 14 h/10 h day/night regime and greenhouse temperatures during the experiment ranged from 20 to 37°C. All growth regulator applications were formulated by mixing or suspending the appropriate amount of chemical in 1.0 L of 1.0 mM NaOH with 5.0 mL of Tween 80 surfactant. Application rates were GA3, 1.6, 3.2, 6.4, and 12.8 g a.i. ha^{-1}; NAA, 160, 320, 640, and 1280 g a.i. ha^{-1}; and OBHA, 0.51, 1.02, 2.05, and 4.10 g a.i. ha^{-1}. Plants from each pot were sprayed with a hand-held sprayer at 2 and 4 weeks of regrowth with 20.0 mL of these formulations. Control plants were sprayed with the same formulations minus growth regulator. There were four replicates of each treatment and control. Plants were harvested after 8 weeks of regrowth and morphological measures and in vitro digestible dry matter (IVDDM) were determined.

Results and Discussion

Gibberellic acid significantly increased plant dry weight and decreased the leaf/stem ratio of alfalfa and significantly increased the height and decreased stem digestibility of smooth bromegrass. In alfalfa, the increase in total dry weight followed the observed increase in stem dry weight. The NAA significantly decreased height and dry weight of all components and increased leaf IVDDM in alfalfa. The NAA did not cause any significant changes to growth, yield, or digestibility of smooth bromegrass. The OBHA significantly increased the leaf/stem ratio in both alfalfa and smooth bromegrass, but either decreased or did not improve the total digestibility of both species.

This research demonstrated that forage growth, yield, and digestibility can be manipulated through application of plant growth regulators, but that there is a general trade-off between yield or growth and digestibility as shown in Figures 1 and 2. Gibberellic acid generally increased growth or yield and decreased digestibility, whereas NAA generally decreased growth or yield and increased digestibility.

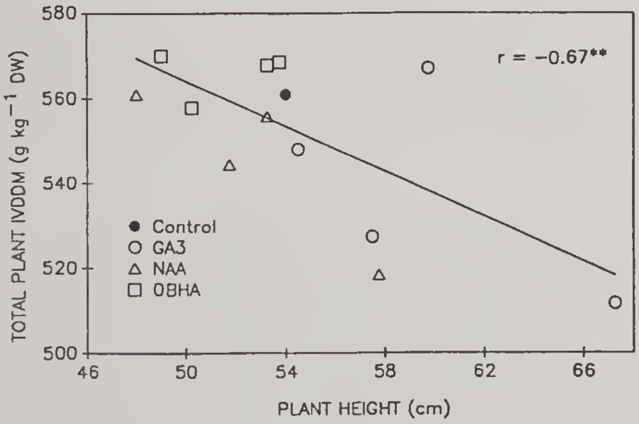


Figure 1. Relationship between total plant IVDDM and plant height of smooth bromegrass after 8 weeks of regrowth as affected by three plant growth regulators.

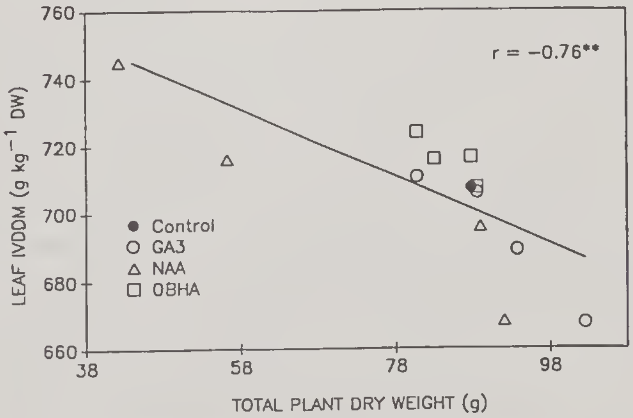


Figure 2. Relationship between leaf IVDDM and total plant dry weight of alfalfa after 8 weeks of regrowth as affected by three plant growth regulators.

ALFALFA VERSUS CORN SILAGE SYSTEMS FOR DAIRY FARMS

L.R. BORTON, C.A. ROTZ and J.R. BLACK

Introduction

Corn silage and alfalfa are two primary roughages for dairy herds in the northern U.S. The literature on corn silage and alfalfa document many studies ranging from whole farm budgeting comparisons to feeding trials. Most studies have not concluded that one type of forage is always economically better than the other. A new comparison is needed due to changes in the dairy industry including higher herd lactation averages and the new interest in nutrient management. A computer model, DAFOSYM, which includes weather risk in forage production and considers the many interactions among farm components can also provide a better analysis of systems than previously possible. DAFOSYM was used to perform a comprehensive comparison of corn silage and alfalfa systems. Specific objectives of the study were to identify the set of inputs that gave the largest net return over feed cost for selected forage ratios of corn silage/alfalfa fed to a dairy herd, and to identify management tradeoffs between strategies with an emphasis on nutrient management.

Methods

DAFOSYM simulates the growth, harvest, storage and feeding of corn silage, corn grain and alfalfa to a dairy herd. The model was expanded to include manure handling and nutrients applied as substitutes for commercial fertilizer. The quantity of manure produced was determined from the nondigestible dry matter consumed. The nitrogen, phosphorous and potassium levels in manure were based on the feed contents with subtractions for that used in milk production and animal growth. Simulation of a representative farm for many years of weather conditions provides a distribution of annual values of farm performance, costs and economic returns. Representative farms with 120, 250 or 400 milking cows were simulated for south central Michigan. Crop yields were adjusted to obtain long-term relative yields of alfalfa, corn grain and corn silage of 9.0, 5.6 and 13.5 t DM/ha, respectively. The forage proportions of corn silage were approximately 0 (all alfalfa), 1/3, 2/3 and 1 (all corn silage) as measured by the annual forage dry matter fed to the dairy herd. Tillable acreage was adjusted to an area that met the corn grain or alfalfa needs of the herd for about 5 out of 6 years. A partial budget analysis was used to compare systems, so only factors which changed between systems were considered.

Results and Discussion

Relative differences in net revenues between selected ratios of corn silage and alfalfa production were not large and represented less than 2% of the average milk and excess crop revenues (Table 1). These amounts could be important to the farm's overall profitability, but are not large enough to encourage a sudden reallocation of resources. A sensitivity analysis showed similar comparisons among forage systems with changes in relative crop yields, lower milk production, constant land area and lower input costs. Changes in herd size showed that the best forage combination may change as the match among machinery, structures and herd size changes. Thus forage recommendations must be farm specific rather than a universal forage ratio for all farms.

Good management was assumed with all systems resulting in equal milk production. All corn silage may require a higher level of management or, at least, different procedures to prevent adverse effects on animal health or milk production. On the other hand, the assumption that manurial nitrogen applied to alfalfa is substituted for nitrogen taken from the air may be optimistic. Applying manure to alfalfa may lead to excess nitrogen leaching into ground water. If this is the case, forage systems, where more than half of the dairy herd's forage need is met with alfalfa, may lead to excessive nitrogen applied to crop land.

Conclusion

There is not a strong economic preference among various ratios of alfalfa and corn silage for the dairy farm. For best use of manure nutrients when only corn and alfalfa are grown, about half of the herd's forage need must be met with corn silage.

Table 1. Various resources, costs and revenues† versus corn silage as a percentage of forage for farms with 120-cow herds at 10,500 kg milk/cow/year and a clay loam (Pewamo) soil.

	Corn silage percentage of forage			
	0.0	31.9	68.5	100.0
CROPLAND & FEEDS FED				
Alfalfa, ha	115	79	39	0
Corn, ha	49	71	99	120
Alfalfa, t DM	782.6	535.5	252.2	—
Corn silage, t DM	—	251.1	547.2	809.6
Corn grain, t DM	50.3	21.5	176.9	112.8
High moisture corn, t DM	184.1	182.7	—	—
Soybean meal, t DM	83.5	100.6	104.9	140.9
Total fed, t DM	1100.6	1091.5	1081.3	1062.7
FEED/CROP COSTS				
Machinery & custom tillage, $	38,405	35,066	36,017	30,149
Fuel & electricity, $	6,813	6,340	5,840	4,862
Storage, $	10,766	12,120	11,462	10,329
Labor, $	20,368	17,996	15,844	11,331
Seed & chemicals, $	16,005	19,133	22,730	25,202
Grain drying, $	1,750	1,481	4,490	3,858
Land charge, $	20,500	18,750	17,250	15,000
Feed purchases, $	19,771	22,954	23,528	31,114
Total feed & crop costs	134,378	133,841	137,161	131,845
CROP SALES & NET REVENUES				
Alfalfa sold, t DM	160.4	104.0	53.1	—
Corn grain sold, t DM	49.4	66.5	102.2	130.5
Total crop sales, $	17,492	14,873	14,358	15,015
Net revenues over costs, $	229,373	227,532	223,696	229,247

†Numbers represent mean averages over 26 years of historical weather.

QUICK-DRYING FORAGE MATS

R.G. KOEGEL, T.J. KRAUS, R.J. STRAUB and K.J. SHINNERS

Introduction

Mats made from alfalfa macerated at the time of mowing and placed on the stubble have been shown in earlier research to dry to a moisture content suitable for baling in less than 6 hours under favorable conditions. Furthermore, alfalfa so harvested proved to have more rapid and extensive dry matter digestibility (generally 10-15% increase) than conventionally harvested material.

Richard G. Koegel

A "second generation" forage mat machine described in the 1991 USDFRC Research Summaries was evaluated. Different configurations of technology had been used to carry out the three major functions of the machine: (1) mowing, (2) macerating, and (3) mat forming in an attempt to simplify, improve reliability, and/or to decrease cost and energy consumption.

Materials and Methods

Comparisons were made between various forage mat parameters when produced by the equipment of the original prototype or that of the "second generation" machine. A total of eight combinations of two mower types x two macerator types x two mat press types resulted. The mat/forage parameters included: drying rate, mat strength, SAI (surface area index, a measure of the extent of maceration), mat dry bulk density, and mat fiber length.

Results and Discussion

Overall results are shown in Table 1. The drying rate was somewhat (drying rate constant $\simeq$ 18% higher) greater for the drum-belt press than for the drum-roll press although drying rate constants for both were low relative to previously achieved values. Mower or macerator types did not affect drying rate. Mat strength, a parameter important in the laying down and picking up of mats without loss, was positively affected by the sickle bar mower and the crushing-impact macerator. Fiber length (that % of the d.m. > 5" in length), a parameter also related to mat strength, was positively affected by the sickle bar mower and the drum-roll macerator. Surface Area Index (SAI), a measure of the extent of maceration, was positively affected by the crushing-impact macerator, but not by either the type of mower or press. Mat dry matter bulk density was greater for the drum-roll press than for the drum-belt press.

Other general observations on the operation of the "second generation" mat machine include:

1. The swing tongue feature functioned well.

2. The press hydraulic drive with feedback control to cause it to rotate at ground speed functioned well.
3. The press did not feed steadily at high roll forces and did not always release the mats intact.

Conclusions

1. The crushing-impact macerator functions very well, is much simpler than its predecessor, and requires less energy.
2. The flail mower significantly reduces mat strength by reducing fiber length and should be replaced by another mower configuration.
3. Press feeding and release characteristics need to be improved to increase mat integrity. A number of alternatives are currently under study.
4. As soon as smooth, reliable functioning of the current machine can be achieved, additional feeding trials should be carried out to determine how the improved digestibility and by-pass protein of mat-harvested forage can best be used in ruminant rations.

Commercial development of machinery for forage mat harvesting continues in Europe. One company will have a limited number of machines for trial by custom operators during Summer 1993 according to reports.

Table 1. Comparison of alternative forage mat equipment/technology.

	Mower type		Macerator type		Press type	
	Sickle Bar	Flail	Drum & roll	Crushing-impact	Drum-Belt	Drum-Roll
Drying rate	0	0	0	0	+	
Mat strength	+			+	0	0
SAI (Surface Area Index)	0	0		+	0	0
Mat density	NA	NA	NA	NA		+
Fiber length % > 5"	+		+		NA	NA

+ Denotes significantly better than the alternative unit.
0 Denotes not significantly different from the alternative unit.
NA Denotes no comparison was made.

ENERGY REDUCTION IN FORAGE HARVESTING: UPWARD CUTTING FORAGE HARVESTER

R.G. KOEGEL, K.J. SHINNERS and M. STELZLE

Introduction

The power requirement for forage harvesting is frequently a limiting factor which determines the rate at which harvesting can be accomplished. Since timeliness of harvest reduces risk of weather damage, a lower power requirement can lead to improved forage. In addition, use of a smaller power unit reduces both capital and operating costs.

Approximately 40% of power required by a forage harvester is used for the blower which functions at approximately 5% efficiency. Past efforts have centered on elimination of the blower by means of inverting the cutterhead and making use of kinetic energy imparted to the forage to convey it to the trailed wagon.

In previous research, an upward-cutting forage harvester was shown to decrease the energy requirement by as much as 34% relative to a conventional cut-and-blow forage harvester. While the movement of forage from the cutterhead into the trailed wagon was considered adequate, it did not have the velocity or the concentrated stream of a cut-and-blow machine. The objective of this research was to evaluate methods of improving material delivery.

Materials and Methods

Two techniques were used to improve material movement from the cutterhead to the wagon: (1) the airflow through the cutterhead was increased by providing about 50% open area in the ends of the cutterhead housing and (2) a radial counter surface was provided a small distance behind each cutting edge against which the forage was compressed as it was sheared and from which it then rebounded. The distance of the counter surfaces behind the cutting edges was adjustable so that the effects of this dimension on material delivery and energy requirement could be studied.

Results and Discussion

The relief dimension from the knife tip to the throwing counter surface has a significant effect on both throwing distance and machine energy requirements (Fig.1). A 3 mm relief produced the greatest throwing distance, but also required the greatest energy. A relief distance of 12 mm produced similar throwing distance to a cut-and-blow harvester but

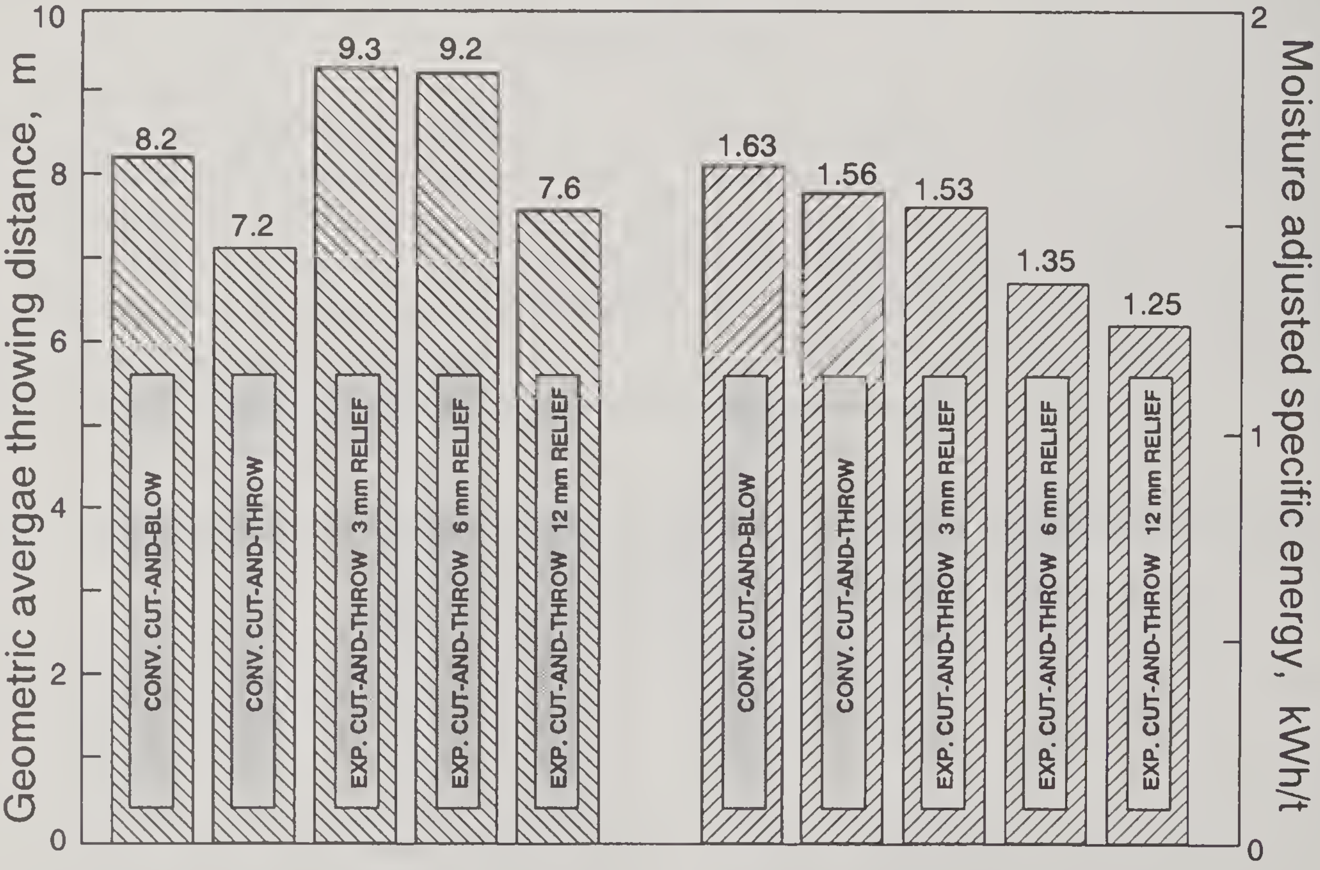


Figure 1. Specific energy requirements and throwing distances for two control forage harvesters and an experimental harvester with three counter surface settings.

required 18% and 23% less energy when harvesting corn and alfalfa, respectively.

Conclusions

The upward-cutting forage harvester equipped with throwing counter surface was shown to significantly reduce energy requirements while having a throwing distance similar to those of cut-and-blow machines. This energy reduction makes possible higher harvesting rates and/or smaller power units. When combined with greater length-of-cut, made practical by bunk silos, the amount of energy reduction could be further enhanced.

DESIGN AND EVALUATION OF A SELF-UNLOADING / WEIGHING WAGON TO EXPEDITE FORAGE HARVESTING RESEARCH

T.J. KRAUS, R.G. KOEGEL, K.J. SHINNERS and R.J. STRAUB

Introduction

Forage harvesting research requires completing many runs on the same day for the purpose of data comparison. This requires large quantities of material to be handled and weighed. In the past, each run yielded samples ranging from 800 to 1000 lb. which were collected in containers and manually weighed. This procedure was labor intensive and typically limited the number of runs per day to 20 or less.

The objective of this project was to design and construct a self-unloading / weighing wagon that could be pulled directly behind the forage harvesting device allowing the material harvested to be immediately collected and weighed. Once weighed, the material could be mechanically transferred to a truck and shipped to the desired location. During the past year a self-unloading / weighing wagon was designed and constructed to allow more research data to be acquired while reducing the labor requirement.

Materials and Methods

The design criteria were to minimize the tare weight of the weighing container while allowing a comparatively large quantity of chopped material to be collected. A maximum tare weight of 1000 lb. and a capacity of 5000 lb. were selected. This capacity would allow five samples, 1000 lb. each, to be harvested before the wagon needed to be emptied. This would reduce the time between runs thus allowing more runs to be performed per day.

Design features of the self-unloading wagon (Figure 1) include: a) a double walled aluminum container that provides high strength and rigidity while minimizing the weight, b) four retractable hydraulic lift cylinders that transfer the weight of the container to the load cells during weighing, c) four 2500 lb. capacity load cells, and d) two hydraulic cylinders that dump the load.

Results and Discussion

The tare weight of the aluminum container was 860 lb. Typically, four samples weighing 600 to 1200 lb., depending on the moisture content and length of cut, could be collected before the container had to be emptied. A maximum variation of ten pounds occurred during repeated measurements of the same load. Typically three individuals could harvest as many as 60 samples during an eight hour period.

Conclusions

The self-unloading / weighing wagon allowed more research to be accomplished per day with less labor. However, changes between harvesting machines were more time consuming due to the extra hydraulic and electrical connections. The wagon provided more repeatable data and required less equipment in the field compared to past procedures. The box functioned properly when filled to maximum capacity.

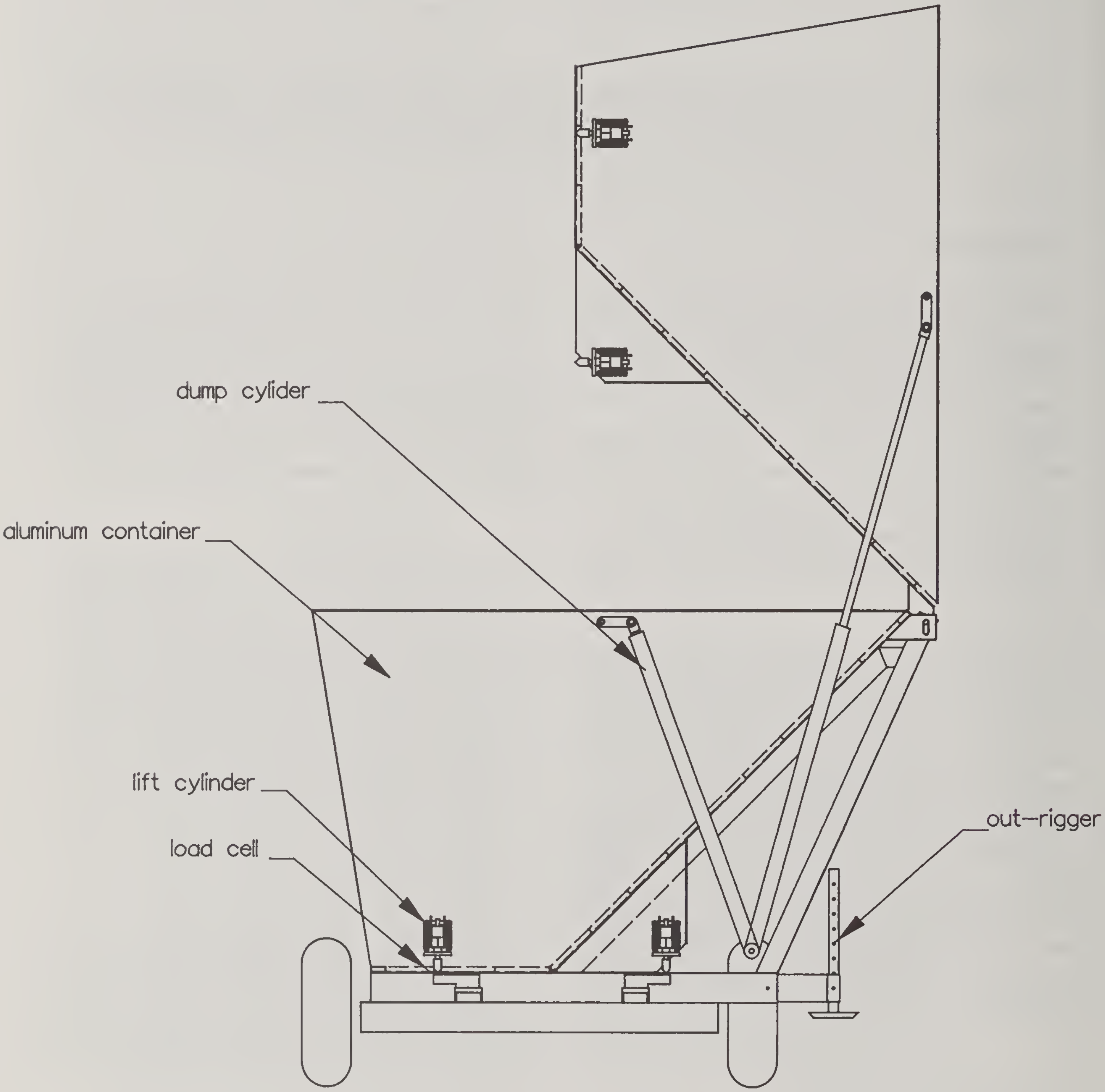

dump cylider
aluminum container
lift cylinder
load cell
out--rigger

Figure 1. Self-unloading / weighing wagon for forage harvesting research.

FRACTIONATION OF ALFALFA JUICE FOR VALUE-ADDED PRODUCTS

R.G. KOEGEL and R.J. STRAUB

Introduction

Forage crops, especially perennial legumes like alfalfa, are renowned for the large quantity of protein and other important nutrients they can produce per unit area. Their production promotes soil conservation and requires low fossil fuel inputs relative to other economic crops. Despite their excellent properties, forages are fed almost exclusively to ruminant animals, because their high fiber content makes them unsuitable for monogastric animals.

The limitations of forage crops can be, at least partially, overcome by wet fractionation. Wet fractionation of green plants such as alfalfa consists of expressing juice from macerated herbage followed by separation of the juice into various high-value constituents, such as protein and/or vitamin-mineral concentrates for feeding to ruminants or monogastrics including humans. The fibrous fraction is normally fed to ruminants fresh, ensiled, or dehydrated. It also has the potential to become a source of ligno-cellulosics for conversion to paper, building material, or bio-fuel.

Biotechnologists have pointed out and demonstrated the possibility of inserting genes into plants which would cause them to produce industrially valuable substances, such as enzymes. Plants, such as forage crops, could thus serve as "factories" for value-added products, useful for biopulping, environmental cleanup, food processing, or domestic cleaning. Very large quantities of enzymes would be needed for these applications and farm-scale production would thus be appropriate and attractive. Being able to do preliminary separation and concentration of valuable fractions at the production site is necessary to avoid excessive transport costs and unmanageably large waste streams concentrated at a centralized processing site.

The protein in plant juice is in two forms: (1) particulate or chloroplastic protein and (2) soluble or cytoplasmic protein. The former is frequently considered to be feed-grade protein while the latter is a high-value, versatile protein. There is thus incentive to separate these two types of protein while attempting to maximize the yield of soluble protein. In the past, protein has generally been separated from the juice by heat coagulation or by pH adjustment. Neither of these methods is considered desirable, however, because they generally deactivate target enzymes, irreversibly change protein, and fail to maximize the yield of the soluble protein. The primary objective of this research project was to develop a low-temperature processing pathway which would clarify the juice by removal of the particulate green material and then concentrate the remaining soluble protein by a factor of at least five to make its transport and subsequent isolation of the target enzyme(s) economically feasible.

A number of techniques were screened to determine their efficacy and suitability for the large-scale separation of protein from plant juice. These included: (1) exposure to a high voltage (20 kV) electrostatic field, (2) direct passage of DC electricity through the juice, (3) direct passage of AC electricity through the juice, (4) freezing followed by thawing, (5) use of flocculants, and (6) centrifugation.

Results and Discussion

Of the techniques screened, some were eliminated because they were ineffective, and others, while effective, were at least temporarily discarded because of perceived technical and/or economic difficulties of scale-up to processing rates as high as 25 ton/hr of juice.

The processing pathway which was finally selected for evaluation consisted of four steps: (1) direct passage of AC electricity through the juice raising its temperature from ambient to 35°C (95°F), (2) 60 min hold time at 35°C, (3) steady-flow centrifugation at a force of 10,000 g to remove particulates, and (4) ultrafiltration through a nominal 10,000 MW filter to obtain a soluble protein concentrate.

The passage of AC electricity through the juice, in addition to warming the juice, has at least two functions according to the literature: (1) chloroplast membranes are disrupted freeing soluble protein from within the chloroplasts, and (2) surface charges on chloroplasts and chloroplast fragments are significantly reduced allowing these particulates to aggregate which greatly facilitates their removal by centrifugation. This aggregation was found to require a certain hold time which was inversely related to hold temperature. At 50°C aggregation was essentially instantaneous whereas at 35°C the hold time for maximum particulate removal was approximately 60 minutes.

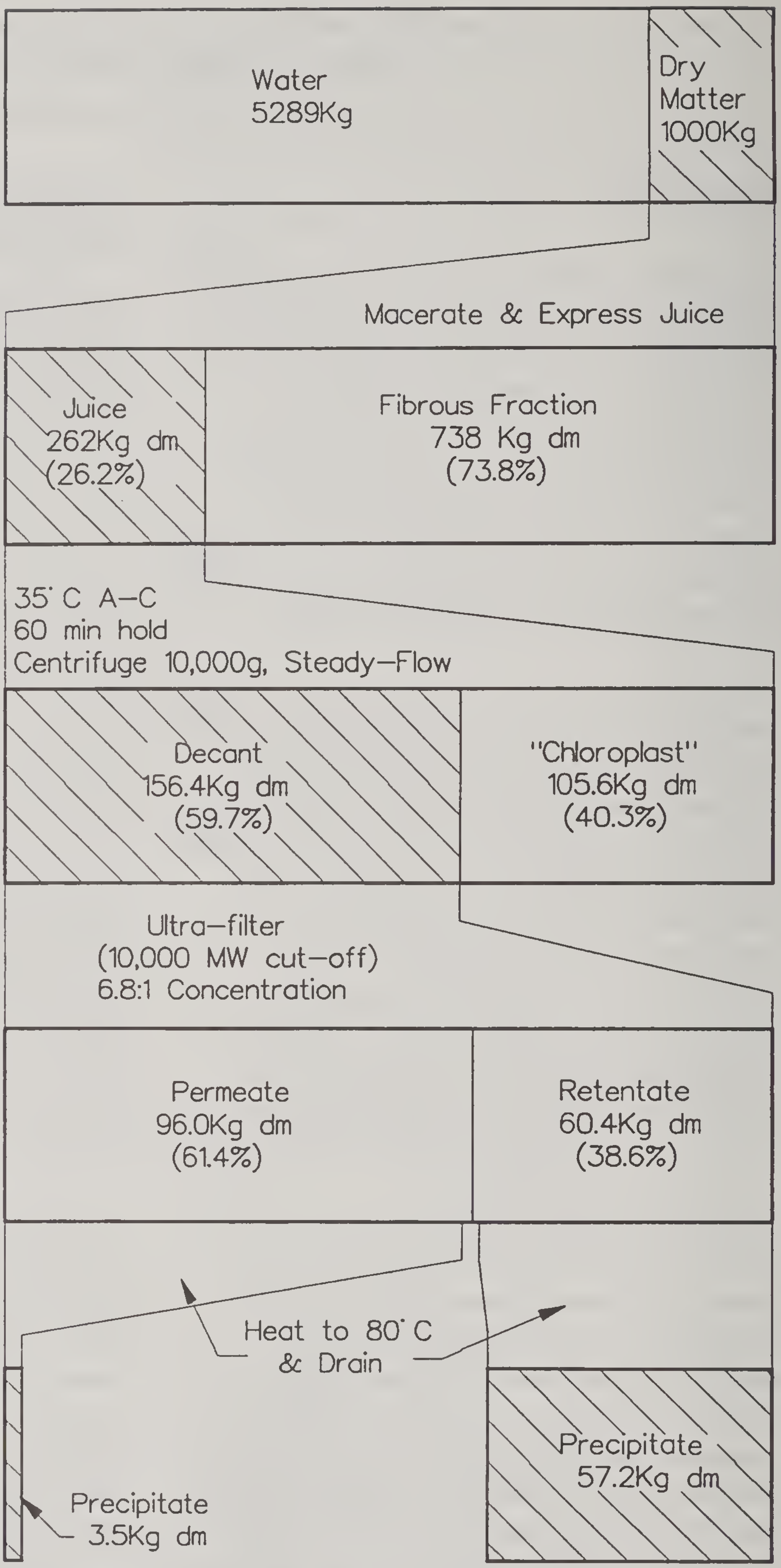

Water
5289Kg
Dry
Matter
1000Kg
Macerate & Express Juice
Juice
262Kg dm
(26.2%)
Fibrous Fraction
738 Kg dm
(73.8%)
35° C A–C
60 min hold
Centrifuge 10,000g, Steady–Flow
Decant
156.4Kg dm
(59.7%)
"Chloroplast"
105.6Kg dm
(40.3%)
Ultra–filter
(10,000 MW cut–off)
6.8:1 Concentration
Permeate
96.0Kg dm
(61.4%)
Retentate
60.4Kg dm
(38.6%)
Heat to 80° C
& Drain
Precipitate
3.5Kg dm
Precipitate
57.2Kg dm

Figure 1. Fractionation of 6289Kg of Alfalfa @ 15.9% d.m., 10/16/92.

After removal of the particulates by centrifugation, the resulting centrate was ultra-filtered to concentrate the soluble protein 5-10 fold. This concentrate could be considered food grade protein after any target enzymes were extracted from it. The green particulate fraction is frequently considered feed-grade protein although valuable constituents such as beta carotene can be extracted from it. Figure 1 shows a mass balance for a typical separation.

Conclusions

1. A four-step process with temperatures not exceeding 35°C (95°F) was achieved to clarify the particulate green protein from the juice and to yield a soluble protein concentrate.
2. This relatively low process temperature should allow recuperation of a wide variety of enzymes with activity unimpaired by heat damage as well as a soluble protein concentrate.
3. At a treatment temperature of 35°C, holding time of approximately 60 minutes is needed to achieve good yields of particulate dry matter ($\simeq$ 40% of juice dry matter) in the centrifuge at 10,000 g for $\simeq$ 2 minutes.
4. The quantity of soluble protein recovered in this study was significantly greater for the 35°C-AC treatment than for a 35°C waterbath treatment. However, this result must be verified under a wider range of maturities, growing conditions, etc.
5. Direct passage of AC electricity through the juice constitutes a convenient and uniform method of heat addition. To raise juice temperature from 20°C to 35°C (68°F - 95°F), the energy requirement is approximately 17.4 kWh/metric ton (15.8 kWh/ton). Part of this could be supplied by waste heat from other processing steps, if desired.
6. At an ultrafiltration flux of 45 liters/m^2-hr, the filter area required to concentrate 22.5 metric tons/hr (25 tons/hr) to a 6:1 ratio would be approximately 420 m^2 (4500 ft^2).

SOLID-LIQUID SEPARATION OF PLANT-DERIVED MATERIALS TO OBTAIN VALUE-ADDED PRODUCTS

R.G. KOEGEL, R.J. STRAUB and J.Y. KIM

Introduction

Solid-liquid separation of plant-derived materials has long played an important role in the food processing, papermaking and distilling/brewing industries. In agriculture, solid-liquid separation has been used for the wet fractionation of forage crops to yield high fiber and low fiber concentrates and for the recovery of undigested feed constituents from manure slurries for re-feeding or for other uses such as bedding.

Since the physical properties of various slurries vary greatly depending on the original material and the processing they have undergone, their separation characteristics also differ. For efficient separation, it is therefore necessary to adapt the equipment and process to the slurry and the desired end product(s). A separation predictive model based on the characteristics of the material would be highly desirable for the design of separation equipment and for selecting values of separation parameters such as pressure and hold time.

Material and Methods

Physical properties of three types of materials (macerated alfalfa, manure slurries, and sweet corn refuse) were determined. These properties included: (1) shear strength, (2) permeability as a function of dry bulk density, (3) particle size distribution, and (4) liquid expression rate as a function of time, pressure, and material thickness.

Three different previously used semi-mechanistic models were evaluated using the observed data. In addition, a finite time increment model was written based on Darcy's Law, the physical properties determined earlier, and the independent pressing parameters.

Finally, based on many observations of liquid expression from plant-derived materials, the moisture content of the fibrous fraction vs. time plot was approximated by two straight lines with the deflection point at 3 seconds. To use this empirical model, the initial moisture content and the moisture content after 10 seconds of pressing in a test apparatus are determined. The slopes of the two lines are functions of the average liquid expression rate during this 10 second period.

Results and Discussion

All of the three semi-mechanistic models evaluated exhibited shortcomings, especially for accurately predicting moisture expression during the relatively short residence periods common to most continuous presses. The finite time increment model based on Darcy's Law predicted moisture content well for most combinations of materials and condition, but not universally. Conditions such as changes in plant physical properties due to maturity and/or environment, or due to the blinding of pores by fine particles are difficult to completely accommodate. The concept of a "characteristic moisture content" such as the moisture content after pressing for a arbitrary time, such as 10 seconds, in a standard apparatus, allowed such differences to be taken into account. Apparently because of this, the 2 straight line model did the best job of predicting the moisture content of a variety of material under a variety of conditions. The R^2 value for goodness of fit always exceeded 0.97.

Conclusions

Many physical properties and pressing parameters influence the expression of moisture from plant materials. This process, however, can be modeled very adequately if the initial moisture and the moisture after 10 seconds of pressing in a test apparatus are determined. Such a model is useful in the design of processes and equipment for solid-liquid separation from plant materials to achieve specific goals.

EFFECTS OF SILO TYPE, SIZE AND SHAPE ON DAIRY FARM ECONOMICS

C.A. ROTZ and R.E. MUCK

Introduction

Many factors must be considered in designing a silage system. A major decision is the type of storage structure. Options include bottom unloaded sealed tower silos, top unloaded stave tower silos and horizontal or bunker silos. Costs, labor requirements, energy requirements and losses are all influenced by the type of structure selected. The number, size and shape of silos are also important considerations. Large silos normally provide a lower cost per unit of silage produced and less loss during storage. With more exposed surface during filling and emptying, losses during these phases may be greater in large silos.

Proper comparison and selection of silage systems require a comprehensive analysis where all factors from harvest to feeding are considered. DAFOSYM provides a tool for performing such an analysis. DAFOSYM simulates the growth, harvest, storage, feeding and use of alfalfa and corn on dairy farms. With the proper input information, the model simulates a representative farm and predicts the performance and costs of the silage system used. By simulating various silage systems for the same farm, the effect of system changes can be compared. The objective of this study was to compare silo types and the effects of changing the number, size and shape of silos on a given farm.

Methods

C. Alan Rotz

Silage systems were compared on representative dairy farms for 25 years of simulated weather for western New York. DAFOSYM parameters were set to describe small- and large-sized farms. The small farm consisted of 30 ha each of alfalfa and corn used to feed 60 milking cows plus young stock. The large farm used 120 ha of alfalfa and 60 ha of corn to meet the forage needs of 250 cows plus young stock. The average annual milk production of the herds was 9,080 kg of milk (3.5% fat) per cow. On the small farm, enough alfalfa silage and hay, corn silage and corn grain were raised to meet the needs of the herd with about 40% of the total forage provided by corn silage. A four cutting alfalfa harvest system was used on both farms. First and fourth cuttings were predominantly chopped and stored as silage with second and third cuttings baled in small, rectangular bales. The large farm was similar except all alfalfa was produced as silage and all corn grain was purchased.

Results and Discussion

On both farms the top unloaded stave silo was most economical. Greater loss in the bunker silo increased the need for purchased corn, hay and soybean meal. Lower storage loss in sealed silos reduced these requirements; however, drier forage was required for sealed structures. Longer field drying periods increased harvest loss, offsetting much of the benefit of reduced storage loss. Variations in machinery, fuel and electric costs were small among silo types. Labor costs were a little higher for the bunker silo due to an extra person needed to operate the packing tractor during filling. Storage costs were similar between bunker and stave silos but substantially greater in sealed silos. Compared to the stave silo, use of the bunker silo on the small farm reduced the net return over feed costs by $1400 per year which was 1.1% of the milk income. A sealed silo reduced net return by almost $3000 per year or 2.0% of the milk income. On the large farm, the annual net return over feed costs for the bunker and sealed silos was over $5000 less than that for stave silos (about 0.9% of milk income).

The use of one large silo was more efficient and cost effective than using two or more smaller silos of equal total capacity. The use of two smaller silos increased the surface area to volume ratio which increased the loss due to oxygen infiltration during storage. Since a smaller surface was exposed during filling and emptying, preseal and feedout losses were reduced. Overall, storage losses were less than 1% greater when two silos were used. With two silos, small shifts in supplemental feed requirements occurred, storage costs increased and the annual net return over feed costs decreased about $1000.

When two silos are used, alfalfa can be separated by quality as it is placed into storage. The highest quality forage can then be fed to high producing animals that best use the higher concentration of nutrients. With this scenario, however, greater preseal and feedout losses occur because both silos are filled and emptied simultaneously and thus at a slower rate. On this particular farm, the increased loss more than offsets any benefit gained by the initial separation of higher quality forage.

With bunker silos, the size and shape affect both the losses and costs. A relatively long and narrow bunker requires greater wall area and thus increases the initial cost. However, the exposed surface is less during filling and emptying which increases the rate of each and thus reduces the loss. A comparison of silo shapes on the large dairy farm indicated that the overall performance, costs and return were similar for the 15 x 92 m and 30 x 46 m silos. With a very wide bunker (46 x 30 m), the reduced storage cost did not offset the effects of increased loss. The feedout rates of the narrow, wide and very wide bunkers were 25, 12 and 8 cm per day, respectively. These results support the general rule that silos should be designed for at least 10 cm of silage removal each day.

Plastic covers often are not used on bunker silos. This reduces the costs for plastic and installation labor and eliminates the inconvenience of dealing with the plastic and tire weights during unloading. Without a cover, storage loss is increased. Simulation of the large farm indicated that the total storage dry matter loss increased from about 9% to as much as 16%. The loss increased supplemental feed requirements, reduced milk production and reduced the net return up to $12,000 per year. The added cost of plastic and labor for the cover ($1400 per year) was small compared to the potential loss. The use of a plastic cover can thus return up to $8 for each additional dollar spent on plastic and labor.

Conclusion

On representative dairy farms in western New York, top-unloaded, stave silos provided the most economical method of storing alfalfa silage. Storing higher quality silage in a second silo separate from lower quality silage was not economical because losses were greater when two silos were filled and emptied simultaneously. Use of a plastic cover on bunker silos returned up to $8.00 for each dollar spent on plastic and labor for installing the cover.

DIRECT-CUT HARVEST AND STORAGE OF ALFALFA ON THE DAIRY FARM

C.A. ROTZ, R.E. PITT, R.E. MUCK, M.S. ALLEN and D.R. BUCKMASTER

Introduction

Alfalfa is normally harvested as either dry hay or wilted silage and both processes often result in large forage losses. One approach to eliminate these losses is to harvest and ensile direct-cut forage. Other benefits include reductions in the number of machine operations, labor and fuel required for harvest. Disadvantages include increased labor, fuel and storage costs from handling more material (more water). Handling of effluent is another problem and a silage treatment is needed to control fermentation in high moisture silage. Despite the disadvantages, direct-cut systems are often proposed to improve forage conservation. A major issue is the economic viability of the process when studied over many years of weather. This type of study is best performed with DAFOSYM, a simulation model of the dairy forage system. A study was initiated to use DAFOSYM to compare the performance and economics of direct-cut and wilted alfalfa silage systems on representative dairy farms.

Methods

DAFOSYM is a comprehensive computer model that simulates alfalfa and corn growth, harvest, storage, feeding and use on the dairy farm. Major resource inputs and system outputs are considered in determining the feed costs and the return above feed costs for the farm. Changes were made to DAFOSYM to model a direct-cut alfalfa silage system. These changes included the parameters and models used to describe direct-cut harvest and the preservation of unwilted silage. A treatment like formic acid was assumed to decrease the forage pH immediately and thereby control fermentation. The cost of the chemical and the added labor and equipment required for the treatment were not included in the analysis. A breakeven treatment cost was determined as the difference in the return above feed cost between the wilted silage and high moisture silage systems divided by the amount of silage treated. This cost represented the highest cost a producer could afford to spend on chemicals, labor and equipment to treat silage with formic acid (or other treatment) without reducing the overall profitability of the farm.

The process was simulated on representative dairy farms. The farms consisted of 100 cows with 50 ha each of alfalfa and corn located near East Lansing, Michigan or Quebec, Canada. A four-cutting alfalfa harvest system was used in Michigan with first and fourth cuttings harvested as silage and second and third baled in small, rectangular bales. Alfalfa silage was stored in two concrete bunker silos with high-quality (less than 43% NDF) silage stored separate from low-quality silage. For wilted silage, alfalfa was cured in the field and harvested after the moisture content dropped below 65% (wet basis). Two strategies were considered for harvesting high-moisture silage. In the first strategy, all silage was unconditionally harvested as direct-cut. The second strategy was to harvest limited unwilted silage only when rain would otherwise delay harvest. Average annual milk production was set at 9,000 kg of milk (3.5% fat) per cow. Prices were set to reflect the long-term relative values for the various farm inputs and outputs in 1991 dollars. A larger farm was also simulated to determine the effects of farm size on the performance and economics of the silage systems.

Results and Discussion

Simulation of the dairy forage system showed that reduced harvest losses with direct-cut silage were largely offset by increased effluent losses from the silo. Little difference occurred in the quantity and quality of forage available to the animals when wilted and direct-cut silage systems were compared.

The economic value of direct-cut silage compared to wilted silage was very poor for dairy farms in central Michigan (Table 1). With no cost for an acid treatment of the high-moisture silage, an economic loss was experienced by the producer due to the small difference in system losses and the greater cost of handling and feeding the wetter material. Assuming a wetter climate such as Quebec, Canada, the economic value of the direct-cut system improved, but the system remained uneconomical compared to wilted silage systems. The economic analysis was relatively insensitive to changes in most parameters and functions assumed in the model. A direct-cut silage system could only be justified over a wilted silage system if feeding of direct-cut silage provided at least a 3% increase in milk production. There is no evidence that this increase could occur.

Conclusion

Development of a system for direct-cut harvest and preservation of alfalfa for the midwestern and northeastern U.S. appears impractical.

Table 1. Comparison of wilted silage and direct-cut silage systems used on a dairy farm with 50 ha of alfalfa, 50 ha of corn and 100 cows located at East Lansing, Michigan.

Annual cost or return	Unit	Wilted silage	Limited high-moisture silage	Direct cut silage
Harvest and feeding machinery cost	$	29,441	29,564	30,965
Energy cost	$	2,722	2,741	2,990
Feed storage cost	$	9,273	9,273	9,273
Labor cost	$	12,394	12,542	13,435
Seed, fertilizer and chemical cost	$	19,617	19,617	19,617
Corn drying cost	$	1,803	1,803	1,803
Feed purchases minus excess feed sales	$	14,514	14,375	12,671
Total feed cost, excluding land	$	89,765	89,914	90,754
Milk income	$	252,000	252,000	252,000
Net return above feed cost	$	162,235	162,086	161,246
Ratio of feed cost to milk income	%	35.6	35.7	36.0
Portion of silage treated	%	0	18	100
Breakeven cost of treatment[†]	$/t DM		-1.83	-3.35

[†] A negative breakeven cost is the cost that must be recovered in addition to the total of all costs required to treat the silage.

HAY PRESERVATION IN VENTILATED BALES

C.A. ROTZ, T.M. HARRIGAN and R.J. TILLOTSON

Introduction

Harvest loss is reduced by baling hay at higher than normal moisture contents (up to 25% moisture). High-moisture hay requires special treatment, though, to prevent increased loss and deterioration of quality during storage. Recently, interest has grown in the use of bale ventilation to enhance hay preservation. Bale ventilation is a process that creates a hole up to 5 cm in diameter through the center of small rectangular bales. Scientific information is needed to quantify the effects of bale ventilation on hay preservation. A study was initiated to compare temperature, dry matter and nutrient losses, and appearance of hay stored in bales with and without a ventilation hole over a range of hay moistures.

Methods

Three trials were conducted during the 1992 growing season where hay was baled in small rectangular bales. To create a hole, a bale ventilator was mounted on the center of the baler plunger. The ventilator consisted of a 5.1 cm diameter cylinder with a conical tip reinforced by four support flanges attached to each side. The 25 cm long spear was bolted to the face of the plunger to enable relatively rapid removal and installation. About 100 bales were baled with and without a ventilation hole in each trial. The goal was to bale hay containing between 20 and 25% moisture.

Immediately after baling, the bales were stacked in a barn with the two treatments in adjoining stacks. Ten bales were monitored in the center of each treatment stack. Thirty gram samples from each monitored bale were dried in a convection oven at 60°C for 72 h to measure moisture content. Bales were weighed and the length was measured just prior to placement in the stack. Bale temperatures and the ambient barn temperature were recorded every six hours throughout the storage period. After three months of storage, bales were removed and the sampling procedure was repeated. Standard wet laboratory procedures were used to measure forage quality in the samples. Quality measures included acid detergent insoluble nitrogen (ADIN), acid detergent fiber (ADF), neutral detergent fiber (NDF) and relative feed value (RFV).

After removing the hay from storage, a visual appraisal was conducted. Three evaluators independently scored five bales of each treatment for mold and color. A numerical score between 0 and 5 was assigned to each bale where 0 indicated no visible mold or very green color and 5 represented heavy amounts of mold or very dark color. Data for each trial were compared using an analysis of variance with bale samples treated as replicates in the error term. Forage quality measures for each treatment following storage were compared to each other and the initial forage quality measures using general contrasts.

Results and Discussion

Bale densities varied between the control and ventilated treatments (Table 1). In the first trial, the density control on the baler was intentionally not changed between treatments in an attempt to obtain similar densities. After baling, ventilated bales were found to be about 15% less dense. Assuming a uniform density throughout the bale, the hole alone would reduce the overall density less than 2%. In the second trial, the density control was adjusted between treatments to obtain similar overall bale densities. In the wetter hay of trial 3, baler plugging was encountered when the ventilating device was used. A relatively low density had to be used to maintain proper baler operation.

Hay heating and appearance were not affected much by the use of bale ventilation (Table 1). The dry hay of the first trial remained near the ambient barn temperature. In the other trials, the average temperature over the first month of storage was 11 to 15°C above ambient with little difference between hay treatments. In trial 3, there was a trend for better color in ventilated bales ($P < 0.1$). This small difference was likely due to the much lower bale density for this treatment. Mold and color scores for all other conditions were similar between control and ventilated bales (Table 1).

In trials 2 and 3, 40 to 50% less dry matter loss was observed in ventilated bales ($P < 0.1$). Since the loss is primarily non-fiber constituents, the loss difference should have caused a difference in final fiber concentrations. Differences in ADF and NDF concentrations were not found. Ventilated bales averaged 11% less ADIN ($P < 0.05$). A difference in ADIN (heat damaged protein) is normally explained by differences in heating during storage. Since temperatures were similar among treatments, this does not provide a logical explanation in these trials. Crude protein concentrations were not affected by storage conditions.

Conclusion

Bale ventilation did not provide significant reductions in hay temperature, dry matter loss and moldiness or improvements in hay quality and color. An unexplained lower amount of acid detergent insoluble nitrogen was found in ventilated bales.

Table 1. Hay moisture content and density before storage, dry matter loss and temperature during storage and hay appearance after storage in bales with and without a ventilation hole.

Trial	Treatment	Initial moisture (%)	Dry matter density (kg/m^3)	Temperature		Dry matter loss (%)	Appearance*	
				Max. °C	Mean °C		Mold	Color
1	Control	16.2	157	22	18	1.4	0.0	1.7
	Ventilated	16.0	138†	23	18	1.9	0.0	1.7
2	Control	21.1	147	48	33	3.5	1.6	2.7
	Ventilated	21.6	144	48	33	2.1	2.0	2.3
3	Control	25.0	123	48	30	3.3	1.7	2.1
	Ventilated	23.6	95†	47	29	1.7	1.7	1.7

*Score of 0 equals no mold or very green hay and 5 equals very moldy or very dark hay.
†Significantly different from the control hay by general contrast ($P < 0.05$).

NET-, PLASTIC- AND TWINE-WRAPPED LARGE ROUND BALE STORAGE LOSS

T.M. HARRIGAN and C.A. ROTZ

Introduction

Over the past several years an increasing number of dairy farmers have adopted large round bales as their preferred harvest method for dry hay. Large round bales generally require less time and labor for harvesting and handling hay compared to small rectangular bales. Direct storage costs are also low when bales are stored outside, but storage losses can be high. Weather has a major effect on these losses, so the effects of storage method on forage losses vary widely across locations. An experiment was conducted to study large round bale storage losses in Michigan. Losses were compared among four storage methods: twine wrapped stored in a shed, twine wrapped stored outside, plastic wrapped stored outside and net wrapped stored outside with all bales elevated on pallets.

Methods

Three round bale storage trials were conducted with alfalfa hay. Three bales (1.2 m long and 1.2 m in diameter) were used as experimental replicates for each storage method in each trial. Bales were stored in rows about one meter apart with 30 cm between adjacent bales. Prior to storage, each bale was weighed and sampled. Samples were collected from the surface to a depth of 10 cm and from 10 cm below the surface to the bale center at a depth of 61 cm. Four samples were collected at each depth from both sides of the bale. Upon removal from storage, each bale was weighed and sampled again. Four samples were collected at each depth from the top, bottom and both sides (32 samples per bale). All samples were dried at 60°C for 72 h and ground to pass through a 1 mm screen. Samples were analyzed for crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent insoluble nitrogen (ADIN) concentrations.

A one-way analysis of variance was used to detect treatment differences within individual trials. Mean separation was done using Tukey's Honestly Significant Difference procedure at a probability level of less than 0.05. Across trials, the experiment was analyzed as a randomized complete block, two factor factorial. The effects of storage treatment, trial and the interaction of treatment and trial were evaluated.

Results and Discussion

Averaged over all trials, dry matter loss in the bales stored inside (6.0%) was less than in the bales stored outside ($P < 0.05$). With outside storage, losses were similar between net-wrapped and twine-wrapped bales (Table 1). Dry matter loss in outside stored plastic-wrapped bales (9.6%) was significantly lower than both net-wrapped (16.3%) and twine-wrapped bales (16.5%). Precipitation trapped in bales stored without a cover caused a large increase in moisture content in the outer layer and a small increase inside the bale. The added moisture likely increased microbial respiration which caused greater loss of nonstructural carbohydrates. The average loss in unprotected bales (twine-wrapped and net-wrapped bales stored outside) was very highly correlated to the number of degree days the ambient temperature was above freezing during the storage period.

Most of the difference in loss among storage methods occurred in the outer 10 cm layer with similar losses inside the bales. Large increases in fiber concentration in the outer layer indicated large losses of nonstructural carbohydrates in unprotected bales. Across all storage methods, NDF increased about 2 to 4 points as a percent of dry matter, and ADF increased from 1.4 to 2.5 points. Crude protein concentrations in the outer layer and all forage quality measures from the center of bales were similar among storage methods ($P < 0.05$). There was little change in ADIN concentration in the inner portion of the bale indicating little or no heating of hay during storage in all treatments. Increases in NDF, ADF and ADIN in the outer layer were likely due to increased microbial activity in the more moist outer portion of the bales. Leaching of soluble cell contents by precipitation on the outer layer may have contributed to the loss as well.

The average hay quality in the whole bale was calculated considering that 35% of the bale mass was contained in the outer 10 cm layer of the bale (Table 1). Hay quality was similar between bales stored inside and those stored outside with a plastic wrap. Averaged over all trials, this hay dropped 0.5 points in CP, gained 2.7 points in NDF and 2.0 points in ADF with no change in ADIN. This represents a 14 point drop in relative feed value. For hay stored outside without a cover, the changes in fiber content and relative feed value were about double that in the protected bales with little change in the crude protein content and a 1.0 point increase in ADIN.

Conclusion

After 6 to 9 months of storage, twine- and net-wrapped bales stored outside contained more moisture throughout than bales from the other two storage methods. Dry matter loss averaged 6.0% in twine-wrapped bales stored inside and 9.6, 16.3 and 16.5% in plastic-, net- and twine-wrapped bales stored outside, respectively. Under Michigan weather conditions, a significant improvement in dry matter recovery is gained by protecting the bale from moisture with a plastic wrap, but the greatest recovery is obtained with barn storage.

Table 1. Dry matter loss (DML) and full bale estimates of hay quality at harvest and when removed from storage, averaged over three trials.

Storage method	DML	NDF	ADF	CP	ADIN	Relative Feed value
	-----------	% DM	-----------		% N	
At harvest						
All hay	---	46.7	31.9	18.2	10.3	134
After storage						
Twine, inside	6.0	49.1	33.7	17.6	10.3	121
Plastic, outside	9.6	49.6	34.0	17.9	10.3	119
Net, outside	16.3	51.7	35.1	18.3	11.2	113
Twine, outside	16.5	52.6	35.2	18.4	11.2	110

EFFECT OF CHLORINATED WATER ON SILAGE INOCULANT BACTERIA

R.E. MUCK

Richard E. Muck

Introduction

Farm wells are sometimes chlorinated to cure a variety of microbial problems with the water supply. If the chlorine dosage is set properly, the remaining chlorine in the water should be minimal. However, monitoring of chlorine dosage is unlikely to be a high priority for farmers. Because water is used to dilute many commercial silage inoculants, it is important to know how sensitive commercial inoculants are to chlorine being present in dilution water. If chlorine levels caused a 90% or greater decrease in bacterial numbers, inoculant performance would be adversely affected. The objective of this study was to determine if chlorinated water affected the viability of selected commercial silage inoculants.

Methods

Six commercial inoculants (four for alfalfa silage, one for corn silage, one for high moisture corn) were diluted as per instructions on the package with distilled water containing 0, 1, 2, 3 or 4 ppm of added chlorine (as NaOCl). The diluted inoculants were allowed to sit at room temperature for 1 hour. Then the number of lactic acid bacteria in each inoculant solution was determined using Rogosa SL agar.

Results and Discussion

Normal recommendations for killing bacteria are chlorine concentrations of 1-1.5 ppm. As shown in Fig. 1, four of the six inoculants could be diluted with water containing approximately 1 ppm chlorine with less than an order of magnitude (i.e., 1 log unit) decline in bacterial numbers. However, only two inoculants had relatively stable counts above 1 ppm. This wide variation in sensitivity to chlorine is probably due to differences in the inoculant carrier rather than to variation in chlorine tolerance of the various bacterial strains.

Simple tests for measuring chlorine concentration are available through stores selling pool supplies. Although these test procedures are not highly accurate, they can provide reasonable estimates of chlorine concentrations. Based on this study, residual chlorine in dilution water should be below 0.5 ppm to ensure the viability of all the inoculants tested. If the chlorine level is above this, the water should be allowed to aerate overnight or longer to remove excess chlorine before the inoculant is added.

Conclusions

Silage inoculants vary considerably in their tolerance of chlorine in the dilution water. Based on the products tested, farmers should have few problems with inoculant bacterial survival if residual chlorine levels are below 0.5 ppm.

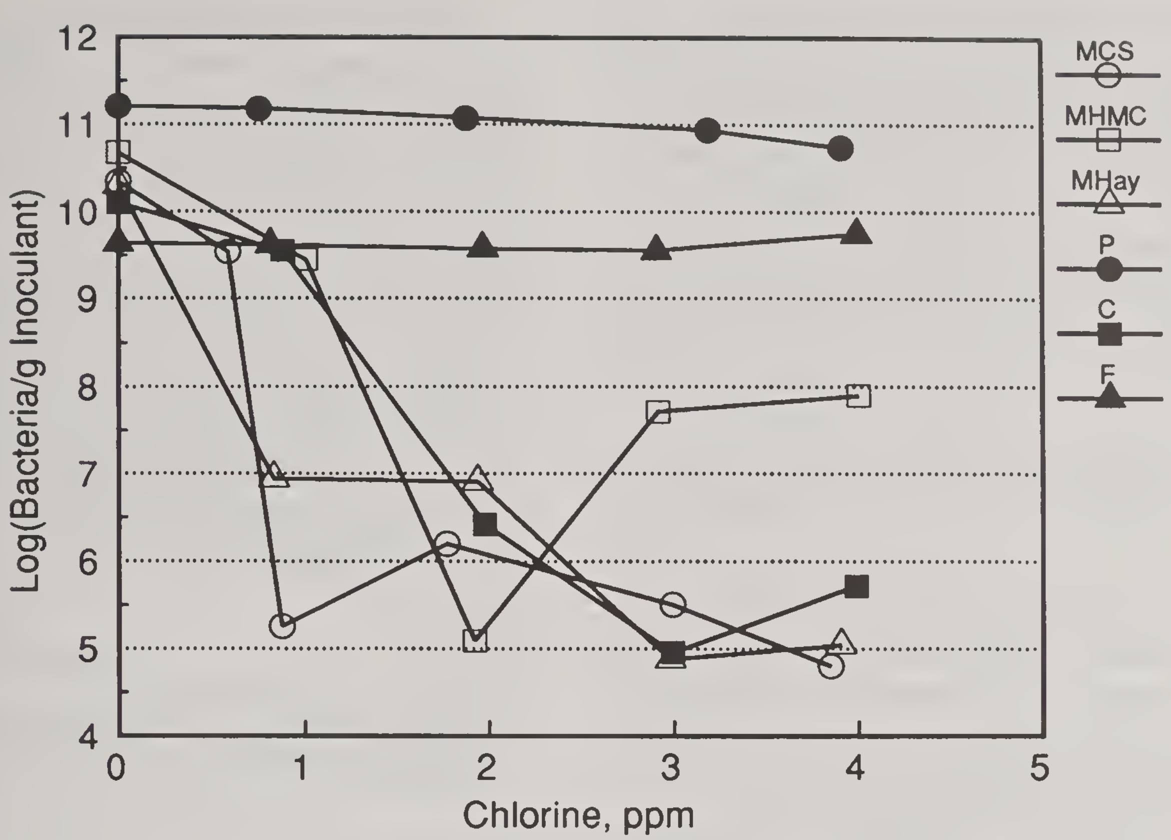


Figure 1. Viable counts in various silage inoculants one hour after dilution as affected by the chlorine content of the dilution water.

AEROBIC DETERIORATION IN CORN SILAGE RELATIVE TO THE SILO FACE

R.E. MUCK and R.E. PITT

Introduction

Corn silage is susceptible to deterioration by aerobic microorganisms in both the silo and the feedbunk. Growth of these microorganisms raises silage temperature and causes loss of dry matter and energy. In most cases, the losses caused by these organisms (typically 5 to 15% DM) are the largest losses incurred during ensiling. Consequently, there is considerable incentive for the farmer to minimize these losses.

In order to develop effective strategies to reduce aerobic losses in the silo, several areas must be understood: mechanisms of oxygen movement into silos, types of microorganisms involved, and conditions under which these organisms thrive. A one-dimensional model of oxygen movement and subsequent microbial development relative to the silo face was previously developed. This model assumed only one group of aerobic microorganisms and that oxygen movement was solely by diffusion. The objectives of this study were to 1) measure oxygen profiles and subsequent microbial development and heating in corn silage relative to the exposed face in laboratory silos and 2) determine the adequacy of the 1-D model to predict the laboratory results.

Methods

Movement of oxygen and subsequent microbial development and heating in corn silage were measured over 4 days at 5, 20, 35 and 50 cm from the exposed face in laboratory-scale (60×15.5 cm diameter) PVC silos. In each trial, 8 silos were packed by hand with corn silage taken from a bunker silo (bulk densities:

602 or 540 kg/m^3). Thermocouples for continuous temperature recording were placed through rubber-stoppered holes in the silo walls at 5, 20, 35 and 50 cm from the open face. Silo walls were covered with 76 mm fiberglass insulation. Oxygen was removed from silos by flushing with carbon dioxide, and then the silos were placed on a laboratory bench for up to 4 d of aerobic exposure. In 4 trials, the silos were oriented as tower silos with the open face at the top; in 2 trials, the silos were laid horizontally as in a bunker silo.

After 1, 2, 3 and 4 d of aerobic exposure, 2 silos were destructively sampled. Initially, gas samples were drawn from each of the four depths and analyzed for oxygen concentration. Then, silage was carefully removed down to 5, 20 , 35 and 50 cm from the face for sampling. Samples were analyzed for moisture, pH, fermentation products, lactic acid bacteria, yeasts, molds, acetic acid bacteria and bacillus spores.

The 1-D model was modified only to account for the specific heat and conductance of the silo wall and radial heat transfer through the insulation. Inputs to the model were the following initial silage characteristics: bulk density, moisture content, pH, lactic and acetic acid contents, and population of the most numerous aerobic microorganism.

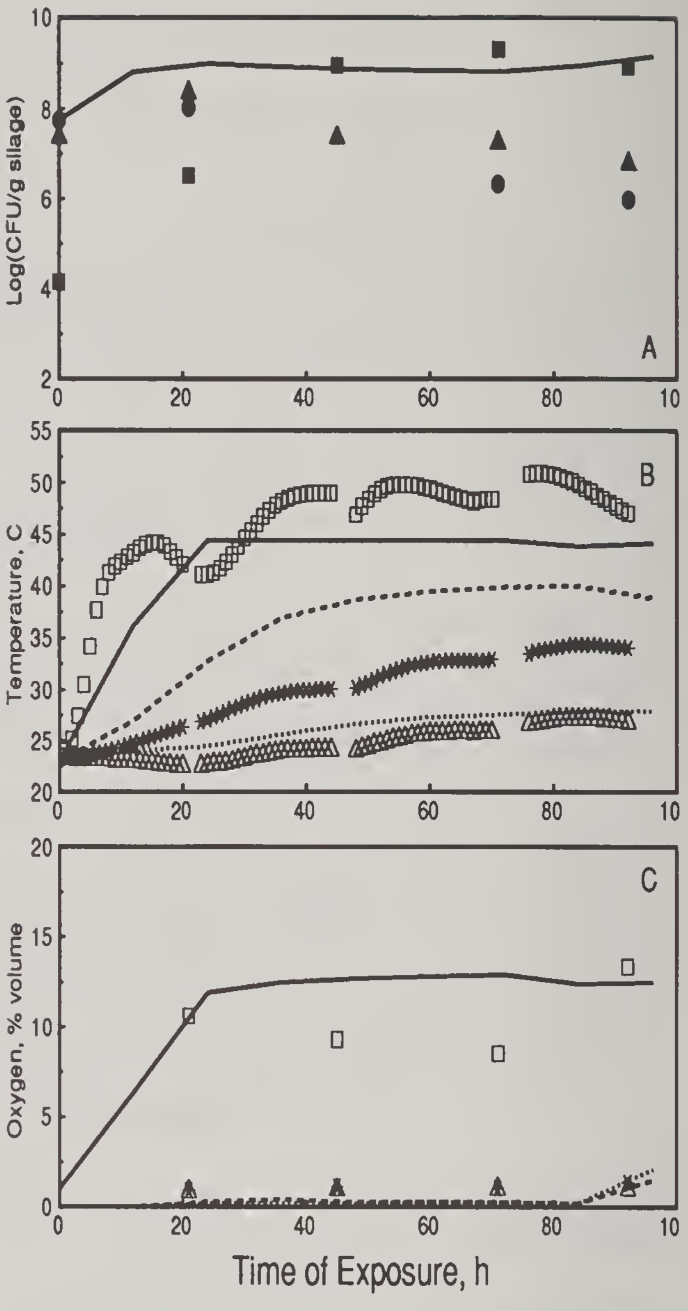


Figure 1. Actual and predicted effects of aerobic exposure on A) aerobic microorganisms at 5 cm, B) temperature and C) oxygen content in corn silage relative to the open face. Symbols: ●, acetic acid bacteria; ▲, yeasts; ■, bacillus spores; ☐, 5 cm; ✶, 20 cm; Δ, 35 cm from face; ——, simulated 5 cm; - - - -, simulated 20 cm; ·····, simulated 35 cm.

Results and Discussion

Actual and simulated results for trial 1 are shown in Figure 1. In all six trials, most microbial activity occurred at the 5 cm level. Acetic acid bacteria initiated heating in all trials although yeasts were significant in trials 1 and 2 (Fig. 1A). Bacilli were not important until silage pH was above 5 and were associated with a secondary temperature rise at the 5 cm level in 4 trials (>30 h in Fig. 1A, B). Oxygen entering the silage at the open face was utilized principally near the face, and temperatures above 40°C were observed only at the 5 cm level. Heating at 20 cm occurred in all trials but appeared to result from heat transfer from the active zone rather than from microbial activity at that depth. Silo orientation had no identifiable effect in these studies.

The 1-D model of aerobic deterioration in silage reasonably predicted oxygen, temperature and microbial numbers in both the vertical and horizontal silos although the predicted progression of deterioration relative to the exposed face was consistently too fast. This is seen in Figure 1 by the fact that the model overpredicted temperature at the 20 cm level. With only one microbial group, the model was incapable of predicting secondary heating at the 5 cm level. Model predictions were improved by reducing by 30% the calculated porosity affecting diffusion. The lack of effect of silo orientation in the actual trials and the similar accuracy of the model in predicting both orientations suggest that diffusion was the primary mechanism of oxygen movement in silage in these laboratory experiments.

Conclusions

These trials confirm Dutch results that acetic acid bacteria as well as yeasts are important initiators of aerobic deterioration in corn silage. This means that strategies to reduce aerobic microbial activity in corn silage must consider both groups. For the first time, bacilli have been identified as agents of secondary heating. Based on our results, molds appear to be the final group involved in aerobic deterioration and indicative of extended periods of deterioration.

The 1-D model reasonably predicted aerobic deterioration relative to the silo face. Predictions could be enhanced by including more than one microbial group. The model also indicated that more research is needed to identify factors affecting diffusion rate in silage.

PROTEOLYSIS IN ALFALFA-SAINFOIN MIXTURES DURING ENSILING

R.E. MUCK and K.A. ALBRECHT

Introduction

Proteolysis in alfalfa silage typically causes soluble nonprotein nitrogen (NPN) contents to be 50 to 70% of total nitrogen at feedout. This reduces the efficiency of nitrogen retention from alfalfa silage by dairy cattle and causes farmers to supplement alfalfa silage-based diets of high-producing dairy cows with less degradable nitrogen sources to optimize production.

In earlier studies, we found that proteolysis during ensiling in legumes containing tannins was reduced relative to that in alfalfa silage by an amount proportional to the tannin concentration. This suggested that mixtures of high tannin-containing legumes with alfalfa might be beneficial in preserving alfalfa protein. Because of its high tannin content, high feed quality and suitability for growth in the northern U.S., sainfoin was selected as the best candidate for such mixtures. Thus, the objective of this study was to determine if the addition of sainfoin to alfalfa at ensiling would reduce proteolysis in the mixture.

Methods

Alfalfa and sainfoin was harvested without wilting and ensiled in minisilos (100 ml centrifuge tubes) in three trials: August 1989, September 1990 and June 1991. In each trial, a 5×2 factorial set of treatments were compared consisting of 5 mixtures (0, 25, 50, 75 and 100% alfalfa on a wet weight basis) and 2 different methods of processing before ensiling (hand chopped; hand macerated). The hand maceration was performed by rubbing the forage between two layers of hardware cloth. This severely disrupted the leaf tissue and split stems longitudinally. In the first two trials, three plots of each forage were harvested, and three silos per treatment were ensiled from each plot, giving 9 silos per treatment. In the third trial, N^{15}-labelled alfalfa from one plot (courtesy of M. Russelle, USDFRC, MN) was used, and 12 silos per treatment were ensiled. All forage was inoculated with a commercial silage inoculant prior to ensiling to minimize differences in fermentation between treatments. The silos were kept at 30°C in a water bath until opening.

Silages were opened after 40 days of fermentation with the exception of the third trial. In trial 3, three silos per treatment were opened after 1, 2, 4 and 40 days ensiling. All fresh forage and silages were analyzed for moisture, pH, total Kjeldahl nitrogen, trichloroacetic acid-soluble nitrogen (soluble NPN), ammonia and free amino acids. Silages were also analyzed for fermentation products. N^{15} analysis of the TCA soluble extract has yet to be performed on the third trial samples.

Results and Discussion

Changes in soluble NPN between the fresh and ensiled forages in each of the three years are shown in Figure 1. In all three years, 100% alfalfa had significantly greater amounts of proteolysis than the 100% sainfoin silages similar to previous results. In general, macerated treatments had less proteolysis than corresponding chopped treatments with the exception of the 1991 40-d silages. Based on other research and the 1991 trial, reduced proteolysis in the macerated silage was most likely due to a faster pH decline in the macerated forage.

The 25, 50 and 75% alfalfa mixtures did not show consistent reductions in proteolysis beyond what would be expected if the two forages had been ensiled separately and mixed at opening. In 1989, proteolysis in each of the chopped mixtures was equal to or greater than that expected from the mixture of the 100% alfalfa and sainfoin silages, suggesting no protection of alfalfa protein by sainfoin tannins. However, some alfalfa protein protection may have occurred in the 25 and 75% mixtures of the macerated forages in 1989. Results for the ammonia fraction were similar to the total soluble NPN. In 1990, neither the chopped nor the macerated mixtures indicated any protection of alfalfa protein with the

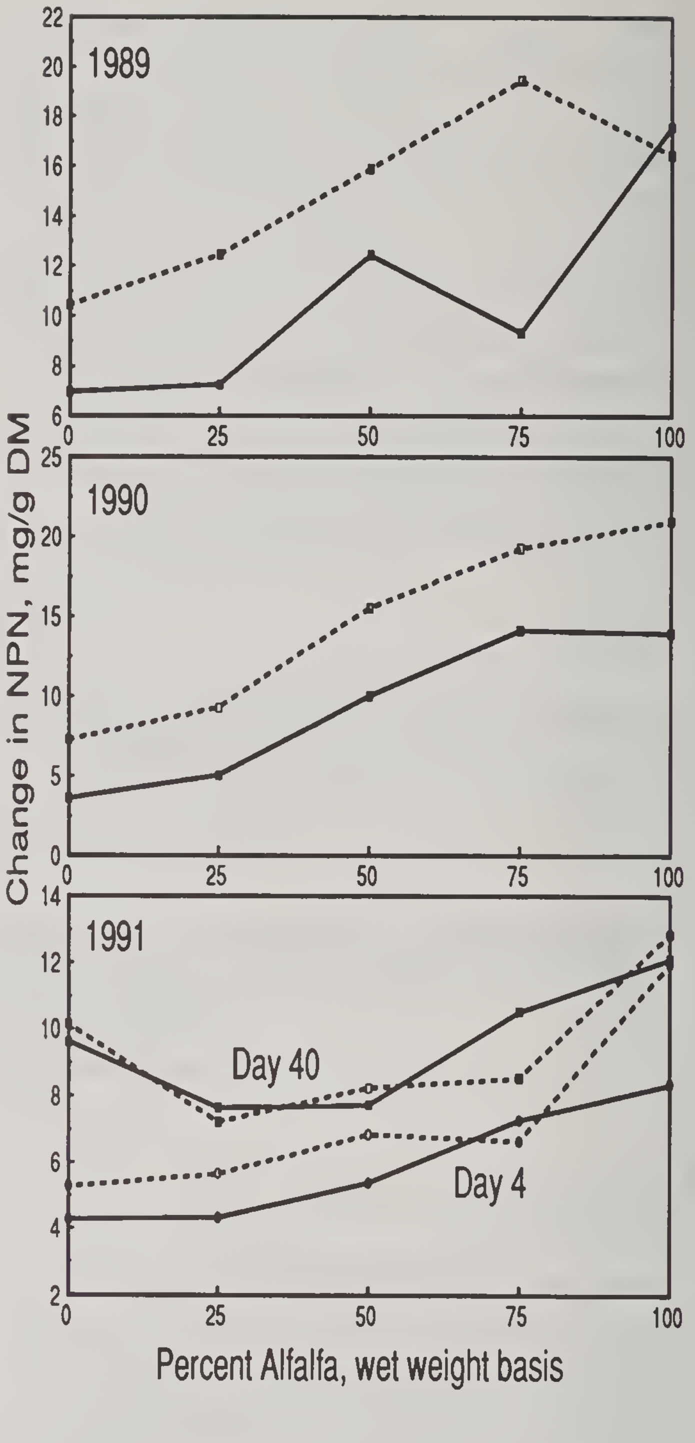


Figure 1. Change in soluble nonprotein nitrogen in alfalfa-sainfoin mixtures during ensiling in each of three years. Hand chopped forage, - - - - -; hand macerated forage, ——.

possible exceptions of soluble NPN in the chopped and macerated 25% alfalfa treatments. In 1991, soluble NPN and ammonia were reduced in all 3 mixtures relative to expected values for both the chopped and macerated silages, particularly in the 40 d silages. In this trial however, soluble NPN inexplicably rose sharply in the 100% sainfoin silages between days 4 and 40 with respect to the other treatments. This sharp rise did not appear to be caused by microbial activity because ammonia did not increase similarly and pHs were 4.0 or less.

Conclusions

Mixtures of alfalfa and sainfoin did not consistently improve the protection of alfalfa protein during ensiling with the exception of the macerated, 25% alfalfa mixture. Consequently, this approach to protecting alfalfa protein does not appear to be practical. These results imply that a more direct application of tannins to alfalfa is probably needed to reduce proteolysis during ensiling.

PRESERVATION OF PROTEIN IN HIGH MOISTURE ALFALFA SILAGE BY DIRECT ACIDIFICATION

D.B. VAGNONI, G.A. BRODERICK and R.E. MUCK

Introduction

Ensiling alfalfa at moisture contents for optimal fermentation (> 60%) results in excessive NPN formation and inefficient N utilization. In an earlier study (Nagel and Broderick, J. Dairy Sci. 75:140, 1992), acidifying alfalfa with formic acid prior to ensiling reduced NPN formation and increased milk production in cows fed alfalfa silage-based diets. It is unknown whether formic acid has any effects on the ruminal degradability of plant proteins beyond acidification. The purpose of the present experiment was to evaluate the effect of formic acid on silage NPN formation and ruminal protein degradation relative to sulfuric acid (merely an acidifying agent) and trichloroacetic acid (TCA; a protein precipitant). Acids were added at levels to bring the forage pH to 4.0.

Materials and Methods

Fourth-cutting, pre-bloom alfalfa was mowed, wilted 48 h (about 30% DM), chopped and transported to the laboratory. A fresh sample also was obtained immediately following mowing and held on dry ice for subsequent freeze drying to evaluate the ruminal protein degradability of the native plant proteins. Chopped alfalfa was divided into 1200 g batches. Each batch was sprayed with the appropriate amount of acid, hand mixed and ensiled in 100 ml polypropylene silos. Silos were incubated in a 30°C water bath. Triplicate silos from each treatment were opened after 1, 2, 3, 4, 7, and 60 d of ensiling. Silages were analyzed for DM, pH, total Kjeldahl N (TKN), non-protein N (NPN), ammonia N (NH_3 N), free amino acid N (FAA N), and peptide bound amino acid N (PBAA N). Samples of d 60 silages were assayed for the rate of ruminal protein degradation (K_d), the potentially degradable fraction (Fraction B), and estimated escape protein using the inhibitor in vitro procedure (Broderick, Brit. J. Nutr. 58:463, 1987). Values for K_d were estimated using the standard, limited substrate (LS) approach as well as by nonlinear regression analysis of the integrated Michaelis-Menten (IMM) equation (Broderick and Clayton, Brit. J. Nutr. 67:27, 1992).

Results and Discussion

Day 60 DM content of all silages was near the target value of 30% (Table 1). The final pH of untreated silage was 4.32; pH of acid-treated silages ranged from 3.82 (sulfuric) to 4.17 (TCA). Following addition of acid, pH of formic and TCA silages was constant over time, while the pH of sulfuric silage was less stable (Figure 1a). The pH decline of sulfuric silage probably was due to some natural fermentation since sulfuric acid has little buffering capacity at pH 4. As expected, protein breakdown was extensive in the untreated silage, NPN accounting for over half of the total N (Table 1). All acids were effective in reducing proteolysis during ensiling; TCA was more effective than sulfuric and formic acids, which behaved in a similar manner. Consistent with previous work, FAA N accounted for the majority of the NPN. Ammonia was the NPN fraction reduced most by acid treatment. Peptides and free amino acids are formed in the silo by the action of plant enzymes, while ammonia is formed by microbial fermentation. Among acid treatments, NH$_3$ N was highest in the sulfuric silage, agreeing with the pH data in suggesting

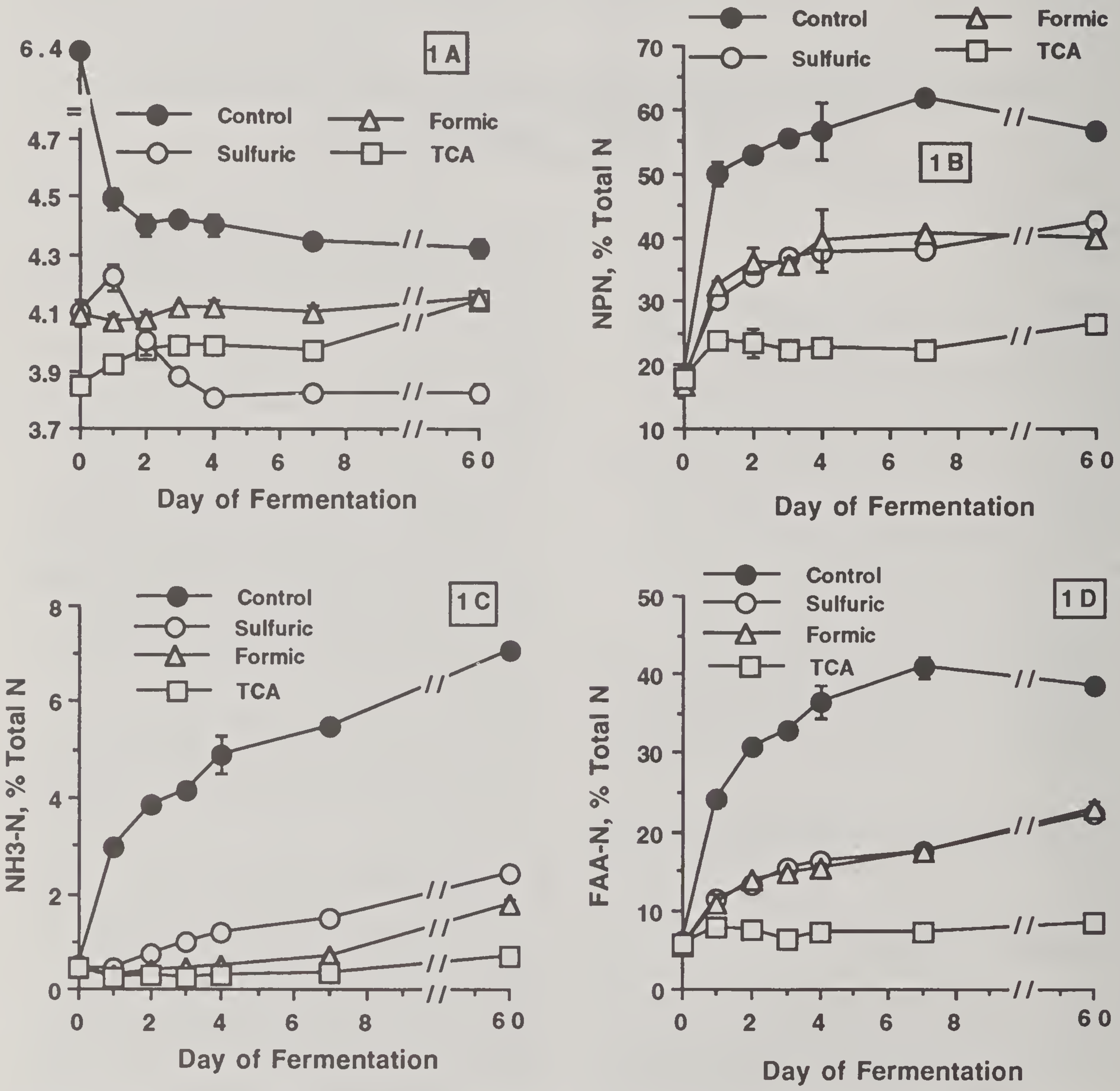


Figure 1. Characteristics of silages over time: a) pH; b) nonprotein N; c) ammonia N; and d) free amino acid N.

that natural fermentation occurred in this treatment.

Because the NPN fraction was reduced, the quantity of Fraction B protein was increased by acid treatment of silage; TCA silage had a Fraction B similar to that of fresh forage (Table 2). Due to the high concentrations of NH_3 N and FAA N (i.e., "background") in control silage, IMM estimates of ruminal protein degradation were unreliable and only LS estimates of K_d are presented. Even LS estimates of K_d for the control silage (Table 2) were on the order of those for resistant proteins such as expeller soybean meal and are quite possibly inaccurate due to the high levels of "background" coupled with a slow release of ammonia and free amino acids from intact protein. Rates of protein degradation were similar among acid-treated silages and were lower than the Fresh forage. Estimated escapes were higher for TCA silage vs. formic and sulfuric silages due to the increased amount of Fraction B protein with TCA.

Degradation rates were consistently higher when estimated by the IMM vs. LS methodology.

Conclusions

Both sulfuric and formic acid treatments gave rise to similar amounts of NPN and larger Fractions B during ensiling as well as similar rates of ruminal degradation and estimated protein escape values. Therefore, it appeared that the previously observed effects of formic acid on the improved utilization of alfalfa silage protein were due simply to acidification of the forage and not to any interactions between formic acid and plant proteins. In support of this observation, treatment with TCA, a known protein precipitant, further reduced protein breakdown during ensiling. Some modification of current ruminal in vitro protein degradation methodology is necessary to adequately measure the escape value of untreated alfalfa silage and other feeds with high "backgrounds" of NH_3 and amino acids.

Table 1. Characteristics of Day 60 alfalfa silages.

				% of TKN			
Treatment	%DM	pH	TKN, %DM	NPN	NH_3 N	FAA N	PBAA N
Control	29.4	4.32	3.08	56.7	7.42	34.1	16.5
Sulfuric	30.0	3.82	3.10	42.6	2.42	18.7	18.3
Formic	31.8	4.15	3.08	39.8	1.77	19.4	20.4
TCA	30.6	4.17	3.14	26.3	.67	7.2	12.8
SE	.3	.02	.05	.9	.10	.4	2.0
5% LSD	.8	.08	.18	2.8	.32	1.4	7.2

Table 2. Degradation characteristics of silages and dry iced fresh forage.

		Kd, h^{-1}		Estimated Escape, %	
Forage	Fraction B	LS	IMM	LS	IMM
Control	47.2	.020	- - -	35.9	- - -
Sulfuric	75.7	.086	.140	31.2	22.8
Formic	75.0	.088	.136	30.8	23.2
TCA	90.2	.087	.126	36.8	29.1
Fresh	91.7	.120	.172	30.7	24.0
SE	.2	.005	.009	1.2	1.0
5% LSD	.7	.016	.032	3.8	3.6

LS=Limited Substrate
IMM=Integrated Michaelis Menten

CHARACTERIZATION OF PROTEOLYTIC ACTIVITY IN ALFALFA AND RED CLOVER

B.A. JONES, R.D. HATFIELD and R.E. MUCK

Introduction

Proteolysis during ensiling in legume forages typically will degrade 40 to 80% of the forage protein. Supplementation of this degraded protein is required when legume silages are fed to high producing dairy cows. This cost could be averted if protein was not degraded or degraded to a lesser extent in the silo.

Alfalfa, the most common legume forage grown and ensiled in the midwest, has the highest extent of proteolysis among the forage legumes, whereas red clover is much lower. Both forages are of similar protein content, but the reason for the lower extent of proteolysis by red clover is unknown. Research was undertaken to characterize the proteolytic activity of alfalfa and red clover.

Material and Methods

Red clover and alfalfa leaves were ground in liquid nitrogen and suspended in buffer (50 mM phosphate, 10 mM ascorbic acid, 5 mM EDTA, pH 7.0) at a ratio of 1 g leaves to 3 ml buffer. The slurry was filtered, centrifuged (20,000 x g, 25 min.), decanted and the supernatant was collected and stored on ice until used.

Proteolysis was measured using ribulose 1,5-bisphosphate carboxylase (RBC) as the substrate (1 mg protein / ml buffer). The buffers used were 50 mM acetate for pH 5.5, 50 mM phosphate for pH 7 and universal buffer for monitoring pH optimum. One-tenth ml of legume extract was added to 1 ml of the RBC solution. Enzyme assays were incubated at 30 or 37°C, and enzyme activity was halted by adding 0.5 ml of 15% trichloroacetic acid to precipitate the undegraded proteins. Free amino acids (AA) in the supernatant were measured by a ninhydrin assay. Temperature and pH stability of the proteolytic activity were determined by pre-incubating the forage extract at the designated pH or temperature for set time points. Aliquot of the extracts were removed and proteolytic activity was determined at pH 5.5 and 30°C.

Results and Discussion

The overall rate of RBC degradation by red clover and alfalfa extracts was not significantly different (0.34 ± 0.018 and 0.31 ± 0.031 micromoles AA released / mg protein-hour, respectively). A rapid pH decline occurs during ensiling and could influence proteolytic activity of legume forages. Yet the pH optima and stabilities of red clover and alfalfa proteolytic activities were similar in the pH range of

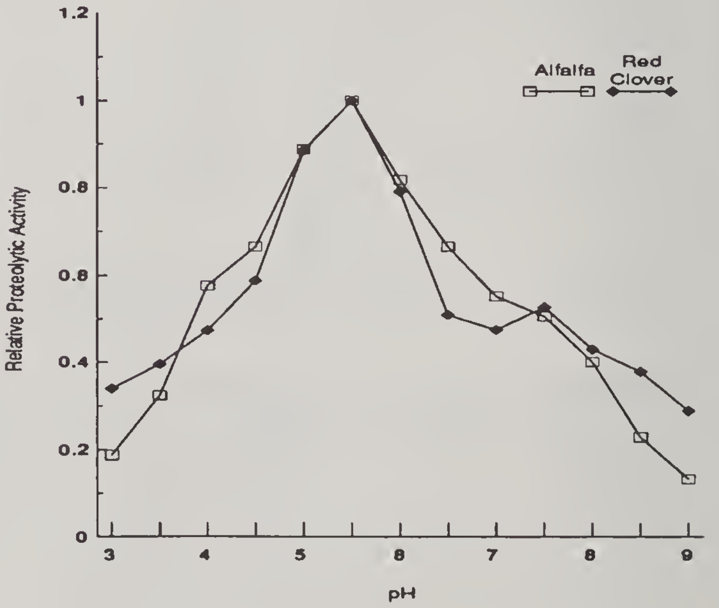


Figure 1. Effect of pH on proteolytic activity of red clover and alfalfa extracts. Substrate was ribulose 1,5-bisphophate carboxylase (1mg/ml) and the reactions were incubated at 37°C for 2 hours.

3 to 6 (Figures 1 and 2). Red clover, after 24 hours of incubation, had slightly more proteolytic activity at pH 4 and 5 than alfalfa and equal proteolytic activity at pH 5.5. These data suggest that the difference in proteolytic activity between red clover and alfalfa is not related to pH effects.

Temperature stability of the plant proteases could also affect proteolysis in silage. However temperature stability of proteolytic activity was similar across the two forages (Figure 3). If anything, red clover activity tended to be more stable. Differences in temperature stability are not the reason for reduced proteolysis in red clover silages.

Conclusion

Proteolytic activity of red clover and alfalfa extracts had similar rates, pH optima and pH and temperature stabilities. The lower extent of proteolysis in red clover silages than alfalfa is not due to differences in the inherent proteases of the two legumes.

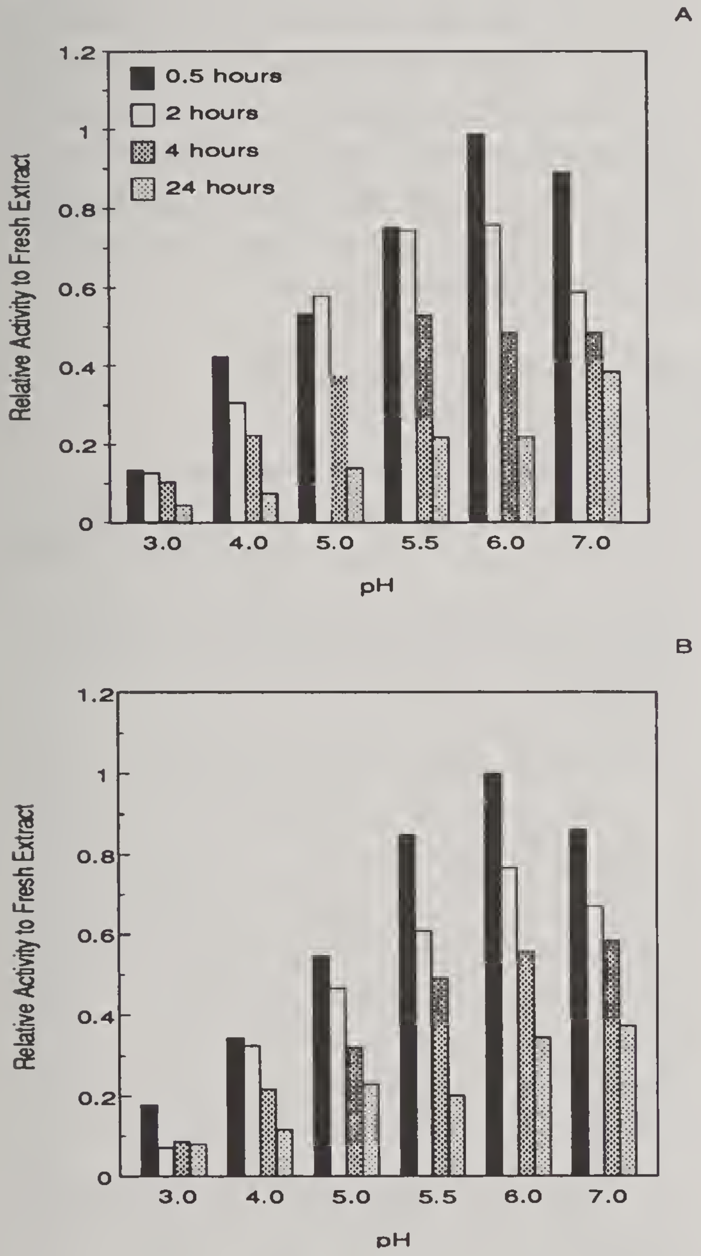


Figure 2. Stability of proteolytic activity of alfalfa and red clover extracts incubated at different pH. An aliquot of the extracts was removed at designated time points and the residual proteolytic activity was measured using ribulose 1,5-bisphosphate carboxylase as the substrate (1 mg/ml) incubated at pH 5.5 and 30°C for 2 hours. A) alfalfa; B) red clover.

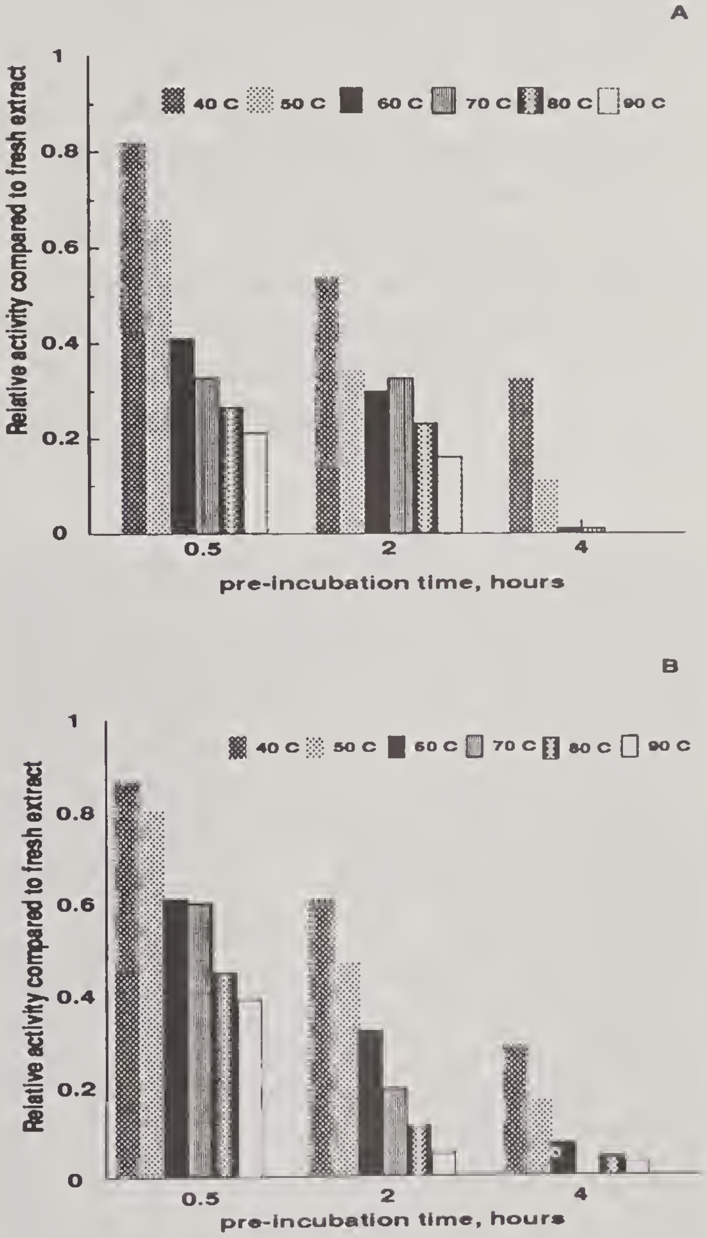


Figure 3. Stability of alfalfa and red clover proteolytic activity incubated at different temperatures. An aliquot of the extracts was removed at designated time points and the residual proteolytic activity was measured using ribulose 1,5-bisphosphate carboxylase as the substrate (1 mg/ml) incubated at pH 5.5 and 30°C for 2 hours. A) alfalfa; B) red clover.

ENZYMATIC BROWNING OF RED CLOVER EXTRACTS

B.A. JONES, R.E. MUCK AND R.D. HATFIELD

Introduction

Enzymatic browning of plant tissue occurs as the result of injury. Polyphenol oxidases (PPO) are responsible for the reaction and may be associated with the defensive mechanisms of the plant. There are several classes of oxidases and their division is dependent on the types of phenols oxidized, but all oxidases will oxidize diphenols. The oxidized phenols could covalently bind to and crosslink other compounds.

Red clover will brown extensively when harvested or when an aqueous extract is prepared from leaves. Little information is available on polyphenol oxidases found in leaves, especially soluble polyphenol oxidases. Research was undertaken to develop a method to monitor soluble oxidase activity in legume leaves and to screen common legume forages for oxidase activity.

Material and Methods

Legume leaves were ground in liquid nitrogen using a coffee grinder. Three ml phosphate buffer (50 mM, pH 7.0) was added for each gram of leaves. The slurry was filtered through cheesecloth and centrifuged on a microfuge. Supernatant was collected and tested for soluble PPO and self-browning activity.

Polyphenol oxidase was measured using 10 mM catechin (a diphenol that is a common substrate of PPO) as the substrate in phosphate buffer. Control was 10 mM catechin and 10 mM ascorbate in phosphate buffer. Legume extract (0.1 ml) was added to the phenol buffer preparations. Enzymatic reaction was incubated at room temperature for 10 min and stopped by the addition of ascorbate to a final concentration of 10 mM and 20 mM for the enzyme assay and controls, respectively. Distilled water was added to a final volume of 3 ml. Absorbance changes from the control were quantified by reading at 435 nm on a spectrophotometer. Oxidase activity was expressed as absorbance change per g fresh weight.

Prepared legume extracts (1.0 ml) were allowed to set at room temperature for 10 min. to determine self-browning of the legume extract. Controls had ascorbic acid added to a final concentration of 10 mM. Reaction of the assay and control was halted by adding ascorbic acid to a final concentration of 10 and 20 mM, respectively. Samples were diluted as indicated above and absorbance was read at 435 nm. All PPO and self-browning activity measurements were replicated.

Results and Discussion

Red clover extracts contained extensive oxidase activity which was not found in alfalfa extracts (Figure 1). This activity was halted by 10 mM ascorbic acid and by 3 mM 2,3-naphthalenediol, an inhibitor of polyphenol oxidases.

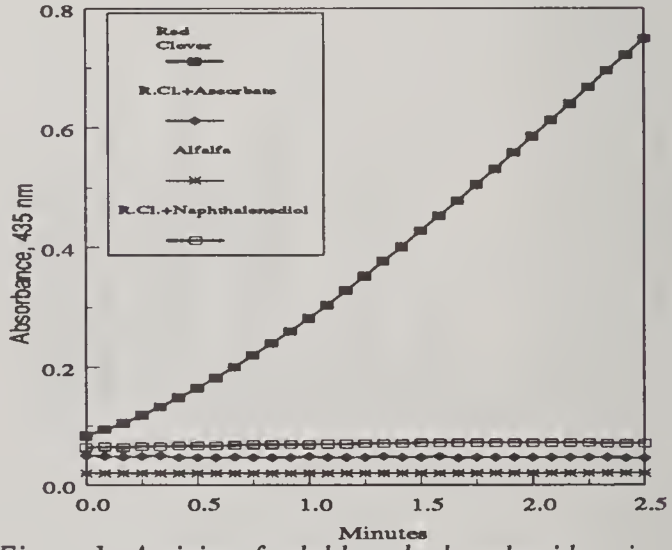


Figure 1. Activity of soluble polyphenol oxidase in prepared leaf extracts of alfalfa and red clover. Substrate was 10 mM catechin in 50 mM phosphate buffer (pH 7.0). Ascorbate and naphthalenediol were added such that the final concentrations were 10 mM and 3 mM, respectively.

Fifteen common legume forages which included varieties of red clover, white clover, kura clover, alsike clover, sanfoin, birdsfoot trefoil, alfalfa (both *M. sativa* and *M. falcata*), lespedeza, crown vetch and cicer milkvetch were screened. Only red clover had extensive soluble PPO activity (48.6 ± 0.57 Abs increase / g leaf) and was capable of self-browning (0.95 ± 0.110 Abs increase / g leaf) in both replicates. Self-browning of the red clover extracts indicates that there are sufficient phenol substrates for PPO activity. Cicer milkvetch did have a small amount of PPO activity (0.51 Abs increase / g leaf) in one replicate but no activity in the second replicate. However, it had no self-browning in either replicate. All other legumes extracts, including the other clovers, had no PPO or self-browning activity in either replicate.

Soluble PPO activity was measured in 18 germplasms and 2 varieties of red clover. All plants had extensive PPO activity and were capable of self-browning. Activities ranged 38.4 to 60.9 and 0.79 to 1.70 Abs increase / g leaf for PPO activity and self-browning, respectively. Reasons for the function of the soluble PPO in red clover are unknown and will require further research.

Conclusion

Red clover extracts contain extensive soluble polyphenol oxidase activity and consequently are capable of self-browning. These activities are not present in other common forage legumes, including other clovers. Reasons for the PPO activity are presently unknown.

CHEMICAL COMPOSITION OF CELL WALLS FROM FORAGE INDIGESTIBLE RESIDUES

H. G. JUNG and D. R. MERTENS

Introduction

The extent of forage fiber digestion is thought to limit intake and utilization of forage by dairy cattle. It has been documented that an indigestible fraction exists for forages fermented in the ruminal environment. The existence of such a fraction suggests that specific structural features of the cell wall pose an insurmountable obstacle to anaerobic microbial degradation of a portion of the wall polysaccharides. This suggests that indigestible residues would be excellent materials for the identification of such structural features. The question arises, are the composition and structure of cell walls in the indigestible residues the same across forage species? Mertens has shown previously that detergent fiber composition of indigestible residues is quite consistent across legumes, C_3 and C_4 grasses. Detergent analyses indicate that forages are similar in the composition to their indigestible residues. Our objective was to examine in detail the chemical composition

Hans G. Jung

of cell walls from forage indigestible residues to determine if the results obtained by detergent analysis can be confirmed.

Materials and Methods

Two legumes (alfalfa and red clover), C_3 grasses (orchardgrass and smooth bromegrass), and C_4 grasses (big bluestem and corn stalks) were selected for study. These forages had been fed to sheep at a maintenance level of intake and fecal samples collected. Both the original hays and the fecal samples were fermented *in vitro* with ruminal fluid for 7 d. The hays, indigestible residue from hay (HayIR), and indigestible residue from feces (FecalIR) were subjected to a total fiber analysis. Samples were treated with enzymes to remove starch and the alcohol insoluble residue was acid hydrolyzed. Individual neutral sugars were determined in the hydrolyzate by high-pressure liquid chromatography (HPLC) and total uronic acids estimated colorimetrically. The non-hydrolyzed residue was corrected for ash content and represented the Klason lignin fraction. Ester- and ether-linked phenolic acids (*p*-coumaric and ferulic acids) were determined by low and high temperature alkaline extractions of alcohol insoluble residues and quantified by HPLC analysis. Pyrolysis-gas chromatography-mass spectral analysis was used to determine the syringyl-to-guaiacyl ratio of the lignin polymer.

Results and Discussion

The total fiber content of the hays and indigestible residues varied among the samples, presumably due to differential amounts of microbial matter from the long-term fermentations. The composition of the total fiber fractions is given in Table 1. For the major components of the cell wall (neutral sugars, uronics and Klason lignin), there was generally a great deal of similarity among the indigestible residues and less similarity of the indigestible residues to their hay sources. There was no significant difference between hays and their indigestible residues for the cell-wall phenolic acids. The composition of the neutral sugar fraction was complex, seven sugars being identified, and the indigestible residues were more similar to their hay source than between forage groups. The composition of the ester-linked phenolic acid fraction shifted toward more *p*-coumaric acid and fewer ferulic acid units in the indigestible residues from all forages. A similar change for etherified phenolics was not observed. The lignin composition of the C_3 grasses was lower in syringyl units than was seen in the other forages, but the indigestible residues did not differ from the hays.

Conclusion

At the level of polymer concentrations, the indigestible residues were quite similar among the forage groups and for hay vs. feces derived indigestible residues. This supports the previous detergent data. At the individual molecular component level, there was less similarity. Klason lignin and ether-linked phenolic acids accumulated in all indigestible residues as expected. These data suggest that the basic types of linkages that determine indigestibility are similar among forages, but the actual components involved differ depending on the species of forage involved except that lignin is the common denominator. *In vitro* indigestible residues should be a useful experimental system for determining the recalcitrant structures in forage cell walls.

Table 1. Composition of total fiber for hays, and *in vitro* indigestible residues derived from the hays and fecal matter from sheep fed the hays.

Forage	Sample	Neutral sugars[ac]	Uronic acids[bc]	Klason lignin[c]	Phenolic acids	
					Esters[a]	Ethers[b]
		- - - - - - - - - - - - -	% Total fiber	- - - - - - - - - - - -		
Legumes	Hay	59.1^{e}	20.2^{d}	20.7	.04	.05
	HayIR	45.0^{fg}	10.5^{e}	44.3	.03	.15
	FecalIR	49.3^{efg}	10.4^{e}	40.2	.02	.09
C_3 grasses	Hay	74.5^{d}	9.1^{ef}	15.3	.66	.45
	HayIR	41.4^{g}	8.8^{ef}	48.8	.27	1.04
	FecalIR	42.6^{g}	5.8^{f}	50.7	.36	.49
C_4 grasses	Hay	76.1^{d}	5.7^{f}	16.0	1.83	.34
	HayIR	49.5^{efg}	7.4^{ef}	41.2	1.27	.65
	FecalIR	53.9^{ef}	5.7^{f}	38.3	1.37	.68

[a] C_4 grasses differ from the other forages ($P < 0.05$).
[b] Legumes and grasses differ ($P < 0.05$).
[c] Hays differ from indigestible residues ($P < 0.05$).
[defg] Means in a column not sharing a common superscript are different ($P < 0.05$).

INITIAL METHODS DEVELOPMENT, FRACTIONATION AND CHARACTERIZATION OF CORN CELL WALL PEROXIDASES

D.E. LUSK and R.D. HATFIELD

Introduction

Plant cell walls are known to contain numerous peroxidases. Unique metabolic roles for these enzymes have been proposed by some researchers. For example, lignin-peroxidase refers to the cell wall peroxidases exclusively (or primarily) responsible for producing monolignol free radicals. Current experimental evidence is insufficient to conclude unique metabolic roles for wall peroxidases. One problem is that these enzymes can utilize a variety of substrates. Consequently, substrates typically used to assess peroxidase activity and roles are convenient, but artificial and structurally dissimilar to the in vivo counterpart. We have hypothesized that specific metabolic roles are plausible and have undertaken a long-term project to investigate and define the metabolic roles of corn cell wall peroxidases, with particular interest in lignin-peroxidase. Our initial work includes the development of extraction and purification procedures, identification and refinement of appropriate assays, and testing of enzyme parameters.

Materials and Methods

Etiolated coleoptiles and pre-tassel (V6-V12) stems of greenhouse grown corn were used to isolate wall-bound peroxidase isozymes. Tissues were homogenized in a Waring blender and then the walls were isolated and washed. Wall proteins were extracted with 3M LiCl or

100mM $CaCl_2$. Evaluation of extracts was based on specific activity with guaiacol (ABS/min/mg protein) of the dialyzed, unconcentrated and concentrated extracts. Concentrated extracts were fractionated using a DEAE anion exchange column. Active fractions were pooled, buffer exchanged, and concentrated. These enzyme preparations were tested for activity and analyzed by isoelectric focusing (IEF) and SDS-PAGE electrophoresis.

Peroxidase activity was determined using guaiacol or 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide as substrates. Assay mixtures contained 1.5 mM guaiacol or 0.25 mM ABTS, 1mM H_2O_2, and 5-100 µl enzyme in a total volume of 500 µl. The quaiacol assay was used to determine pH optima, and calcium and pH stability. Determinations of pH optima and pH stability utilized TRIS-acetate buffer with a pH range from 4 to 8 in half unit steps for optima, and whole unit steps for stability. Guaiacol and 3,3'-diaminobenzidene (DAB) were used for activity staining of IEF gels and to test walls for residual activity after extraction and digestion treatments. The protein concentration of enzyme preparations was determined using the BCA protein assay (Pierce).

Mechanical reduction of particle size and enzyme digestion were used in an attempt to remove residual activity from isolated, salt extracted walls. Walls were ground with mortar and pestle in liquid nitrogen. Ground walls were reextracted with fresh $CaCl_2$ and buffer washed prior to digestion. Two cellulases and 2 xylanases were tested separately on individual wall samples. Salt extracts, washes and digestion supernatants were assayed for peroxidase activity with quaiacol.

Results and Discussion

Calcium chloride extracts had a higher specific activity than LiCl extracts. Fifteen to 30 minutes in extraction solution and subsequent buffer wash was sufficient to release the majority of salt extractable peroxidase activity. Further release of activity was achieved by reduction of wall particle size followed by reextraction with fresh $CaCl_2$. However, these treatments were insufficient to remove all the activity from the walls. Digestion of these twice extracted walls released more enzyme; yet not all activity was removed. The peroxidases that remain in the wall must be covalently bound or are very effectively entrapped in the cell wall.

Fractionation of coleoptile extracts by DEAE column chromatography resulted in 3 active pools (CP1-CP3) whereas stem extracts produced 5 active pools (SP1-SP5). The majority of the total protein and total activity loaded on the column remained unbound (CP1 & SP1). Individual pooled fractions produced multiple bands on IEF gels stained for activity, indicating the presence of isozymes. IEF and SDS-PAGE gels stained for protein also had multiple bands. The CP1 and SP1 pools had active bands on IEF gels in the basic (pH > 8), slightly acidic (pH 5.5-7), and acidic (pH < 5.5) ranges. All other pools had active bands in the acidic range. A minimum of 10 active bands on IEF gels were easily recognizable.

Use of both guaiacol and ABTS as peroxidase substrate has been reported, exalted and debased in the literature. We found that ABTS was more reactive than guaiacol with 2 of the DEAE pools, but less reactive with the other pools. Pools less ABTS reactive have the lowest specific activity and are most difficult to identify as active prior to pooling.

We therefore consider guaiacol to be a superior substrate for the identification of corn wall peroxidases. Another factor in assessing activity is that azide either quenches free radicals or inhibits peroxidase activity, especially at low enzyme concentrations.

Peroxidase fractions were more stable when stored in the presence of 5mM $CaCl_2$. The pH stability varied in range between DEAE pools. All of the pools were stable at pH 7, except SP4 which was more stable at pH 6. Thus, we used 50 mM TRIS buffer with azide and 5 mM $CaCl_2$ for peroxidase storage. The pH optimum of SP1, SP2 and SP3 pools was pH 5.5-6, SP4 was pH 5-6, and SP5 was pH 5-5.5. We now do all activity assays with sodium-acetate, pH 5.5 buffer without azide or $CaCl_2$.

Conclusion

The data presented here partially define our model system and were prerequisite to designing experiments that address our hypothesis.

A BIOMIMETIC ROUTE TO LIGNIN MODEL COMPOUNDS VIA SILVER (I) OXIDE OXIDATION

S. QUIDEAU and J. RALPH

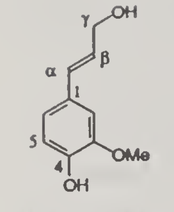
Introduction

The key mechanism in lignin biosynthesis is the oxidative coupling of phenols. Delocalized phenoxyl radicals generated upon phenol dehydrogenation of *p*-hydroxycinnamyl alcohol monomers and intermediate lignin units couple in a variety of ways to build up the lignin polymer. This free radical-mediated dehydrogenation is thought to be initiated by peroxidases-H_2O_2 and/or laccase-O_2. The peroxidases-H_2O_2 system is commonly used for the preparation of synthetic lignins, or dehydrogenation polymers (DHPs). These DHPs, as high molecular mass lignin model compounds, constitute a unique tool to determine the available chemical pathways followed during lignification processes. Elucidation of the DHP and lignin structures requires the synthesis and characterization of adequate low molecular mass model compounds and strongly relies on the application of NMR spectroscopic techniques.

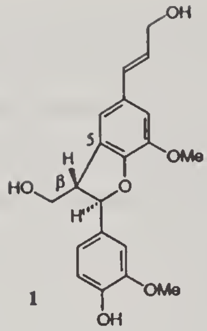
Modelling an important lignin substructure phenylcoumarans such as β-5-coupled dehydrodiconiferyl alcohol (**1**) have been the targets of a few synthetic reports, but the schemes proposed remain tedious and low-yielding. A facile access to coniferyl alcohol (see The 1991 Research Summaries) led us to look for a more expedient route to the β-5-coupled dehydro-dimer **1**. This dimer was needed for derivatization with *p*-hydroxycinnamic acids in the framework of our investigations on the *p*-hydroxycinnamic acid/lignin complexes in grass cell walls. An oxidative coupling approach was chosen, using silver (I) oxide as a one-electron oxidant, and provided a biomimetic route to additional lignin model compounds by variation of the reaction conditions. For example, β-O-4-type benzyl alcohols and/or non-cyclic benzyl aryl ethers were obtained upon *in situ* trapping of β-O-4 quinone methide intermediates.

Method

To a solution of coniferyl alcohol (see Table 1) was added silver (I) oxide (1.5 eq) and the reaction mixture was stirred at ambient temperature until disappearance of coniferyl alcohol, as monitored by TLC. The products were isolated by standard chromatographic



Coniferyl Alcohol



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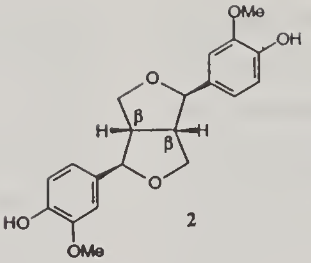

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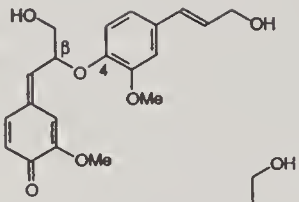

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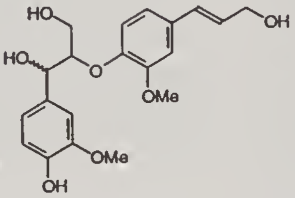

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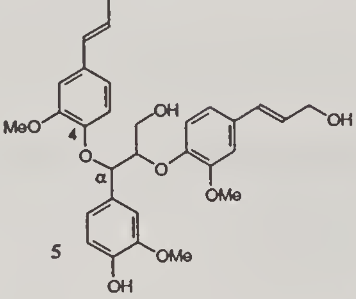

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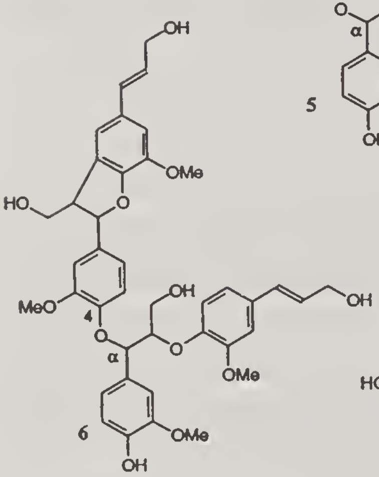

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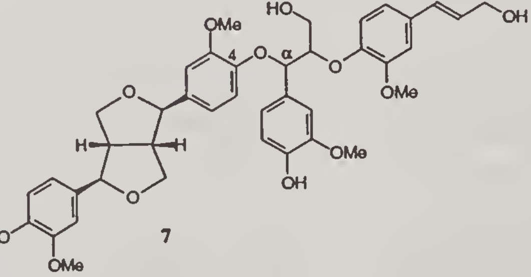

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techniques and analyzed by NMR spectroscopy.

Results and Discussion

The Ag_2O oxidation of the lignin monomer (*E*)-coniferyl alcohol in methylene chloride (CH_2Cl_2), gave β-5-coupled dehydrodiconiferyl alcohol (**1**) in yields up to 50% (Table 1). This good-yielding one-step synthesis of **1** constitutes an improved oxidative coupling method for the preparation of this important dilignol and a convenient alternative to the previously reported multi-step synthesis. About 5-10% of the non-cyclic benzyl aryl ether (**6**), formed via phenol addition of the dilignol **1** to the β-O-4 quinone methide **3**, was also isolated. When the reaction was carried out in aqueous solution at pH 3, the only product resulting from nucleophilic addition to quinone methide intermediates was the benzyl alcohol **4** (18%), indicating that, under these acidic conditions, water is the preferred nucleophile attacking the reactive carbonium ion obtained by protonation of the quinone methide **3**. At pH 5.3, only traces of **4** were obtained, whereas significant amounts of the *erythro* isomers of the non-cyclic benzyl aryl ethers **5** (24%), formed upon addition of coniferyl alcohol to **3**, and **6** (10%) were isolated. Under neutral conditions (pH 7-8), besides dehyrodiconiferyl alcohol (**1**) and pinoresinol (**2**), the *erythro* isomers of benzyl aryl ethers **5**, **6** and **7** were isolated.

Conclusions

During the silver (I) oxide-mediated oxidative coupling of coniferyl alcohol in neutral aqueous and non-aqueous solutions, quinone methide intermediates[3] generated *via* β-O-4 radical coupling rapidly led to the formation of non-cyclic benzyl aryl ethers **5-7**. These α-O-4 linked structures, which are potentially important branching units in lignins, were the predominant products resulting from nucleophilic attack onto β-O-4 quinone methides. The addition of water to β-O-4 quinone methides, which is predominant during lignification, became important only when catalyzed by acids. The synthesis of the phenylcoumaran dehydrodiconiferyl alcohol **1** *via* silver (I) oxide oxidation of coniferyl alcohol in methylene chloride is an improved and convenient oxidative coupling procedure for the preparation of this important lignin model compound.

Table 1. Silver (I) oxide oxidation of coniferyl alcohol.

Conditions	Reaction time	Isolated compounds(yield %)					
		1	**2**	**4**	**5**	**6**	**7**
CH_2Cl_2	24 h	50	5	-	traces	6	-
pH 3[a]	4h	24	14	18	-	-	-
pH 5[a]	5 h	24	13	traces	24	10	traces
pH 7-8[b]	5 min	17	10	traces	14	15	4-5

[a] 1:2 acetone:citrate buffer (2.0 M citrate).

[b] 1:1 acetone:water

HIGHLY STEREOSELECTIVE METHOD FOR PREPARATION OF MODEL CELL WALL LIGNIN/HYDROXYCINNAMIC ACID COMPOUNDS

J. RALPH and R.F. HELM

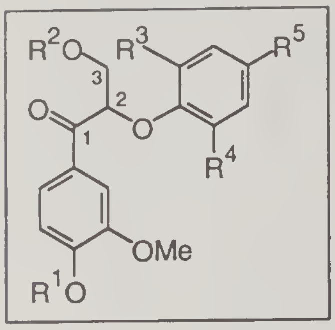
Introduction

Our studies concerning the regiochemistry of hydroxycinnamic acid attachment to lignin and polysaccharides in forage grass cell walls are based on obtaining spectroscopic information on appropriate model compounds and extending the data to the native and synthetic lignin-based polymers. Synthetic schemes to produce lignin models and their derivatives containing hydroxycinnamic acids traditionally produce mixtures of isomers from which separation can be difficult. Recently we observed very high stereoselectivity in the reduction of compound **8** with zinc borohydride. An examination of the factors involved has led to synthetic methods of very general and valuable utility in this area.

John Ralph

Methods

Zinc borohydride ($Zn(BH_4)_2$) reductions of compounds **1-11** were typically performed with an excess of borohydride as follows. The carbonyl compound (100 mg) was dissolved in ethyl acetate (3 mL) and cooled to 0 °C. An ethereal solution of $Zn(BH_4)_2$ (0.15 M, 3 mL, previously prepared from zinc chloride and sodium borohydride) was added and the reaction monitored by TLC. When complete disappearance of starting material was noted, the excess borohydride was quenched with aq. NH_4Cl , extracted into ethyl acetate and processed as usual. Complete conversion was usually within 1-4 h and reduction yields were always greater than 90%.



	R¹	R²	R³	R⁴	R⁵
1	Ac	H	H	H	MeP
2	Ac	H	H	OMe	MeP
3	Ac	H	OMe	OMe	MeP
4	Ac	H	H	OMe	AraP
5	Bn	H	H	OMe	H
6	Bn	Ac	H	OMe	H
7	Ac	AcC	H	OMe	H
8	Ac	AcF	H	OMe	H
9	Ac	Ac	OMe	OMe	MeP
10	Ac	TBDMS	H	OMe	H
11	Bn	EE	H	OMe	H

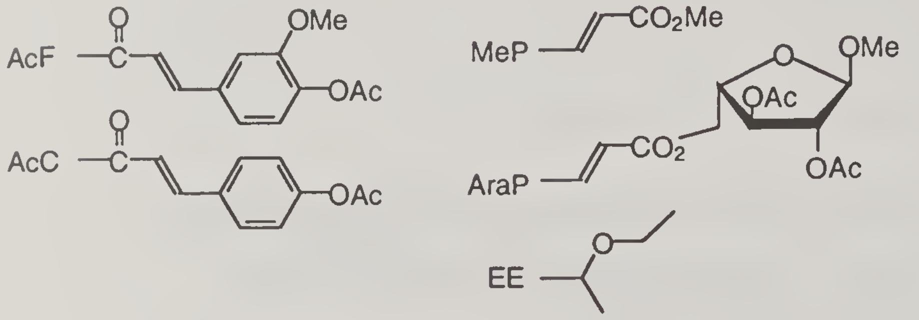


*Figure 1. Compounds **1-11** which were subjected to zinc borohydride reduction. Compounds **1-5** are of type **i**, Figure 2; compounds **6-11** are of type **ii** and show high erythro reduction selectivity.*

Discussion

The stereochemical outcome for the $Zn(BH_4)_2$ reduction of several lignin model precursors is compiled in Table 1. It is readily apparent that the benzoyl carbonyls of α-aryloxy-β-hydroxy systems did not undergo reduction with significant stereoselectivity (**1-5**). The *threo*-isomer was slightly favored in all cases with the α-(2,6-dimethoxyphenoxy) substituent (3) affording a 70% *threo*-product. However, acylation, alkylation, or silylation of the β-hydroxyl and subsequent reduction typically gave the hydroxy compounds with high *erythro*-selectivity (**6-11**). The reduction of **9** (the acetylated derivative of **3**) provided an increased proportion of the *erythro*-product when compared to **3**, but was not as highly stereoselective.

Previous studies concerning the reduction of lignin model compounds similar to type **i** (Figure 2) with a methyl group instead of the β-hydroxyl have shown *erythro*-selectivity with $Zn(BH_4)_2$ as long as the α-aryloxy 2-position was not occupied by a bulky substituent. Complexation with the α-oxygen and/or the α-aryloxy 2-methoxyl oxygen has been proposed to explain the high *erythro*-selectivity. Thus lignin model compounds differ from the compounds described previously in that complexation and subsequent hydride attack occur on the same face as the α-substituent (**v**). For compounds

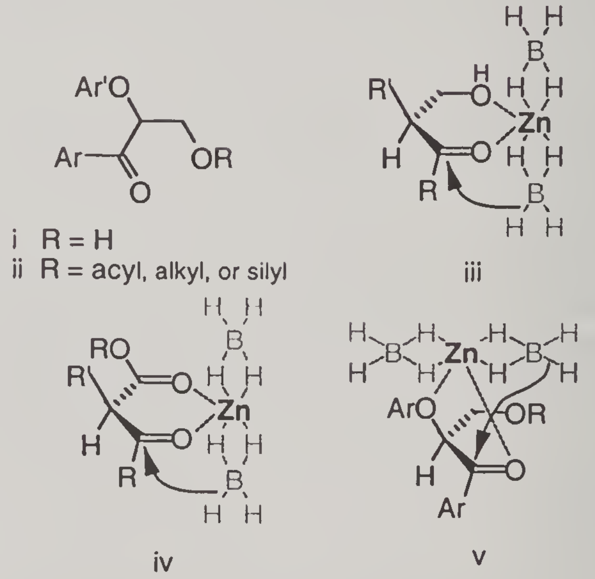


*Figure 2. Compound types **i** (β-hydroxy) and **ii** (β-substituted) and the possible zinc borohydride coordination and hydride transfer complexes; complexes **iii** and **iv** give rise to threo products, complex **v** to the erythro isomers.*

of type **i**, competition between the α-aryloxy oxygen (**v**) and the β-hydroxyl (**iii**) for complexation with the zinc cation would afford the intermediate *threo*/*erythro* reduction ratio observed for **1-5**. The *threo*-isomer predominated as complexation with the hydroxyl was more influential than with the α-aryloxy substituent. Extending this rationale to compounds of type **ii**, protection of the β-hydroxyl group has allowed the α-aryloxy group to exert more of an influence over the course of the reduction. Complexation and hydride attack occur on the same face as the α-substituent providing the *erythro*-isomer. That the protective group on the β-hydroxyl is not complexing with the zinc cation (**iv**) is demonstrated by the reduction of **10** and **11**, where TBDMS and 1-ethoxyethyl groups were employed; high *erythro*-selectivity was observed in both cases, as with the acyl groups in compounds **6-9**.

In conclusion, the highly stereoselective reductions obtained with $Zn(BH_4)_2$ allow for significantly improved lignin model synthesis, providing an efficient route to *erythro* dimers and higher oligomers, utilizing the same general synthetic pathway previously developed for *threo*-selective model syntheses.

Table 1. Zinc Borohydride Reduction *threo*/*erythro* Product Ratios.[a]

Compound	threo:erythro
1	55:45
2	52:48
3	70:30
4	60:40
5	64:36
6	5:95
7	5:95
8	5:95
9	14:86
10	3:97
11	4:96

[a] Determined by NMR spectroscopy via integration of the appropriate side chain proton signals (±2%).

SYNTHESIS OF GUAIACYL AND SYRINGYL LIGNINS AND THEIR EFFECT ON CELL WALL DIGESTION

J.H. GRABBER, A. PELL , J. RALPH, S. QUIDEAU and R.D. HATFIELD

This work was partially sponsored by Miner Institute, Chazy, NY.

Introduction

Studies with field grown forages suggest that lignin composition influences the digestibility of forage fiber. It has not, however, been possible to isolate the effects of lignin composition on fiber digestion. This is because lignin, *p*-hydroxycinnamic acid, and structural carbohydrate compositions all vary considerably in forages, making it difficult to attribute digestibility differences to specific cell-wall constituents.

Cell walls from suspension cultured Black Mexican Sweet Corn (BMSC) may be a suitable model system for isolating the effects of lignin composition on fiber digestibility. Although lignification is limited in BMSC, these cultures contain cell-wall peroxidase which is a key enzyme of lignin formation. In a preliminary study, synthetic lignins were formed when wall-bound peroxidase and H_2O_2 (from glucose oxidase/glucose) was used as an oxidative system for forming phenoxyl radicals from coniferyl and sinapyl alcohols. Our objectives were to produce cell walls with a range of lignin compositions (varying ratios of guaiacyl and syringyl groups) and to determine the effect of these lignins on the *in vitro* digestion of cell walls.

Materials and Methods

Cell walls of BMSC were prepared by sonicating cells followed by washing with HEPES buffer (25 mM, pH 7.5), 100 mM $CaCl_2$, and water. Wet cell walls (70 g) were placed in five 500 mL Erlenmeyer flasks and suspended in 120 mL of PIPES buffer (25 mM, pH 6.5) with 2 mM $CaCl_2$ and 2,500 U of glucose oxidase (Sigma Type VII-S). Five solutions containing 150 mg glucose and 120 to 140 mg of *p*-hydroxycinnamyl alcohols were added to the cell-wall suspensions with a peristaltic pump over a 24 h period. The *p*-hydroxycinnamyl alcohols were added in molar ratios of 100:0, 80:20, 60:40, 40:60, and 20:80 of coniferyl alcohol to sinapyl alcohol. Suspensions were mixed for a total of 70 h. Cell walls were then collected on a nylon net and washed six times with 250 mL of water and dried with acetone. Treatments were replicated two times. The quantity of lignin formed was estimated by a modified Klason procedure. Analyses of lignin composition were done by pyrolysis-GC-MS. Cell wall digestion was estimated by monitoring the production of fermentation gasses produced by samples (50 mg) incubated in buffered rumen fluid.

Results and Discussion

Pyrolysis indicated that unlignified walls contained ferulic acid and small amounts of *p*-coumaric acid and guaiacyl derived components (guaiacol, 4-ethylguaiacol, and vanillin). Lignified walls produced pyrograms characteristic of guaiacyl and syringyl lignins in grasses suggesting that the lignins we synthesized were similar to those produced in forage grasses. Pyrograms also indicated that guaiacyl:syringyl ratios varied with the proportion of coniferyl and sinapyl alcohols added to cell walls. Klason lignin contents increased from 8.0 mg g^{-1} in unlignified walls to ~ 80 mg g^{-1} in walls when coniferyl and sinapyl alcohols were added in ratios of 100:0, 80:20, 60:40, and 40:60. These results suggest that all of the *p*-hydroxycinnamyl alcohols added in these treatments were polymerized into lignin. Lignin formation was apparently less efficient when a coniferyl to sinapyl alcohol ratio of 20:80 was used since Klason lignin levels of this treatment were only 62 mg g^{-1} of cell wall. Unlignified walls were rapidly and almost completely digested after only 24 h of digestion (Figure 1.). Lignification reduced cell wall digestion. In contrast, fiber digestion was not affected by lignin composition. Our results indicate that concentration of lignin has a greater impact on fiber digestion than does the composition of lignin. Further work is underway to evaluate the impact of lignin composition on fiber digestion over a wide range of lignin concentrations.

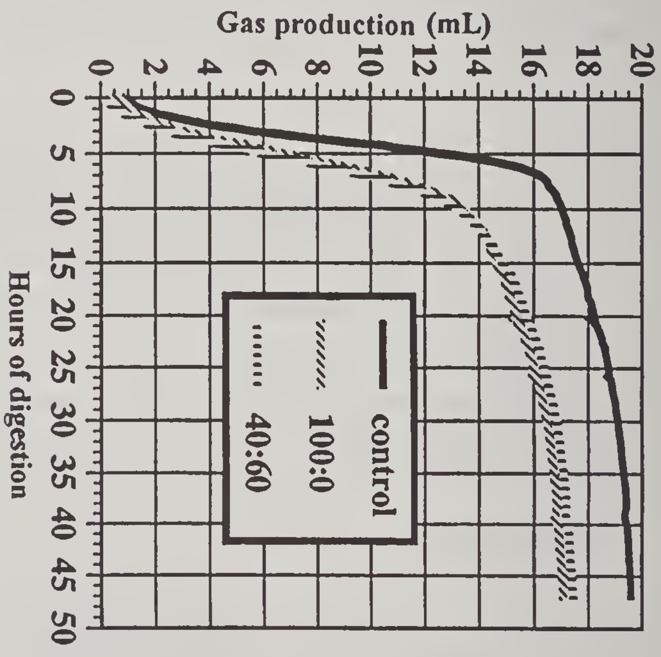


Figure 1. In vitro gas production by rumen microbes on unlignified (control) and synthetically lignified cell walls from suspension cultured corn. Cell walls were lignified by the peroxidasic polymerization of coniferyl and sinapyl alcohols added in ratios of 100:0 and 40:60.

Conclusions

Correlative studies with field grown forages suggest that fiber digestion is influenced by lignin composition. We synthetically lignified walls of suspension cultured corn to determine the effect of varying ratios of guaiacyl and syringyl lignins on fiber digestion. Pyrolysis indicated that synthetic lignins formed in our system were similar to those produced in forage grasses. Lignification reduced cell wall digestion compared to unlignified controls, whereas lignin composition had no effect on fiber digestion.

DETERMINATION OF THE REGIOCHEMISTRY OF *p*-COUMARIC ACID ON CORN LIGNINS

J. RALPH, R.D. HATFIELD, R.F. HELM and S. QUIDEAU

Introduction

Hydroxycinnamic acids are well established components of the cell walls of grasses and legumes that are implicated in cross-linking of cell wall polymers. Our initial efforts have been directed at model studies to prepare and characterize expected cross-linking structures, and a detailed examination of the active incorporation of hydroxycinnamic acids into synthetic lignins (see the 1991 and 1992 Research Summaries) to determine what types of structures are chemically possible and likely to occur. This work is the first to utilize the acquired data to determine, unambiguously, the way in which these structures are incorporated into plant cell walls. The regiochemistry (that is, the position of attachment) of hydroxycinnamic acids has strong and direct implications regarding the biochemistry of its attachment and helps elucidate plant biochemical mechanisms.

In grasses, which contain significant amounts of *p*-coumaric acid (*p*CA), it has been shown that most of the *p*CA is esterified to the lignin. However, its position of attachment to lignin has not been rigorously demonstrated. In bamboo, Higuchi's group deduced from chemical studies that 80% of the *p*CA was esterified to the γ-position and 20% to the α-position. In light of some ambiguities in interpretation of these results and the fact that each site requires fundamentally different and divergent biochemical pathways (attachment as α-esters requires that the free acidic form of *p*CA is incorporated 'opportunistically' into the polymer by attacking intermediate quinone methides, whereas attachment as γ-esters implicates the active incorporation of preformed coniferyl alcohol-*p*CA esters into the free-radical polymerization process), we sought to unambiguously establish the attachment site(s) of *p*CA on lignin.

Methods

A corn sample was selected and fractionated as described in the following report. Approximately 45% (the D-LPPC fraction) was readily soluble in 9:1 acetone-d_6:D_2O and was used for the NMR studies described. The sample (270 mg) was dissolved in 2.4 ml of the above solvent, and spectra determined on a Bruker AMX-360 NMR spectrometer using a 10 mm normal configuration probe under standard conditions. Phase-sensitive proton-detected short-range C-H correlation (HMQC) spectra were determined using the Bruker's invbtp microprogram acquiring 2K data points in the proton dimension and 128 increments of 320 scans each in the carbon dimension. Cosine-bell apodization was applied in each dimension and Fourier transformation gave a final data

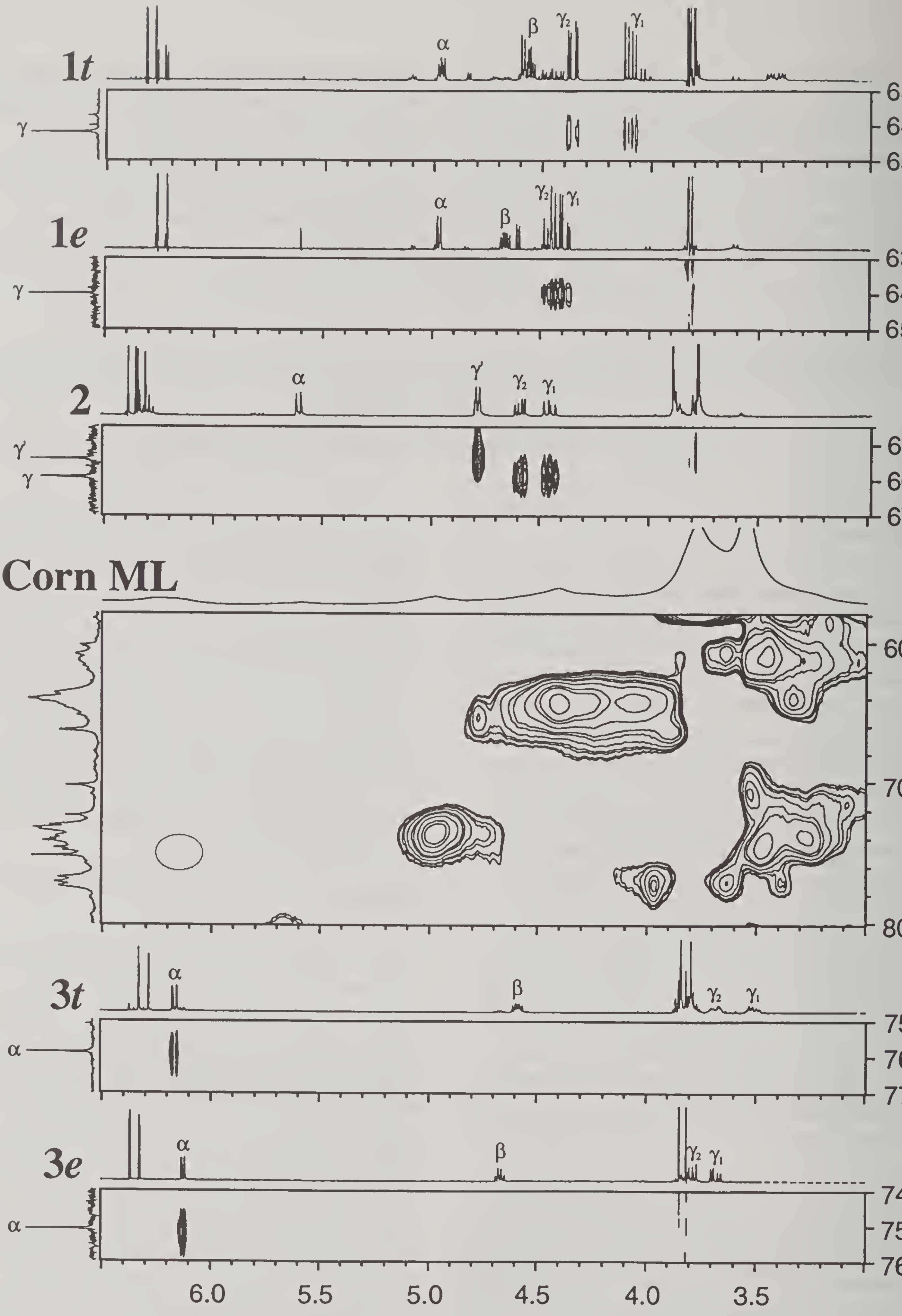


*Figure 1. HMQC (short-range C-H correlation) spectrum of the corn lignin along with appropriate sections for γ-esterified model compounds **1t** (threo) and **1e** (erythro) and **2**, and α-esterified compounds **3t** and **3e**.*

matrix of 1K by 1K real points. Proton detected long-range C-H correction (HMBC) spectra were determined using Bruker's inv4lplmd microprogram with critical long-range coupling delay set at 80 ms (optimized for 6 Hz couplings). 2K data points were acquired in the proton dimension, with 256 increments of 480 scans in the carbon dimension. Apodization by matched Gaussian functions was applied in each dimension followed by Fourier transforming to give a final matrix of 1K by 1K real points. Esters were determined by subjecting the samples to degassed 2M NaOH for 24 hr, extracting in ethyl acetate or ether, and quantitating silylated derivatives by GC as described in the following report. Compound authentications were done using standard compounds and via GC-MS. Synthetic models used for authentication of assignments were prepared by methods too detailed to describe in this format.

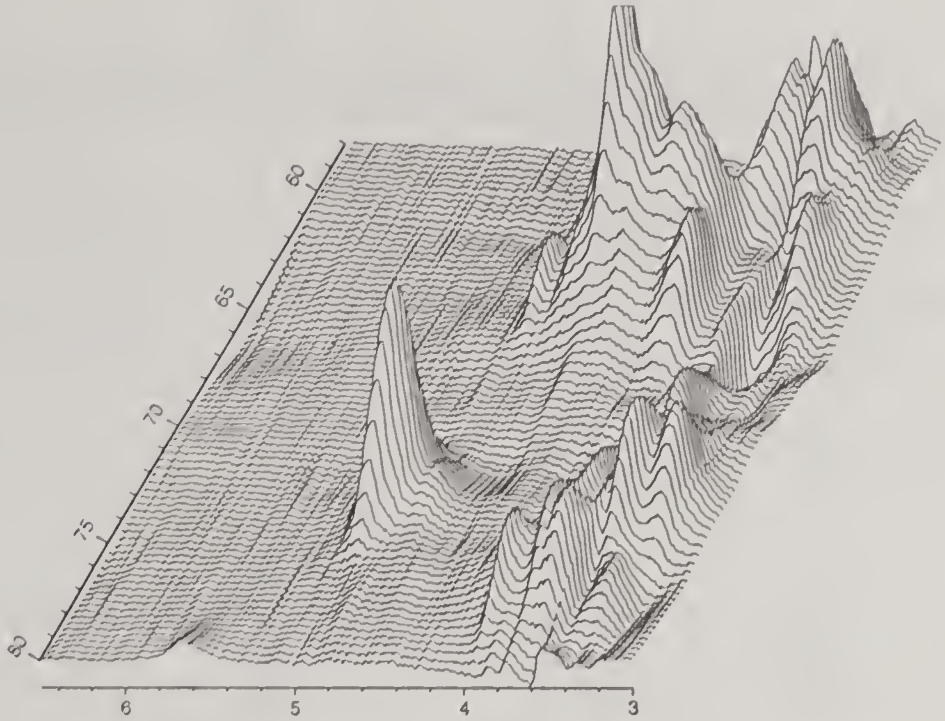


Figure 2. Stacked plot of the corn lignin HMQC spectrum showing that no α-ester correlations are observed above the spectrum baseline.

Discussion

The short-range C-H correlation (HMQC) spectrum, Figure 1, conclusively shows two important features. Firstly, the huge contour relating carbons at 62-68 ppm to protons at 3.8-4.9 ppm shows that the γ-position of the lignin is extensively esterified. This spectrum by itself does not indicate what other molecule is involved in the ester bond. This enormous contour peak is completely absent in samples of, for example, alfalfa lignin (which has very few hydroxycinnamic acids esterified to the lignin) or to a sample of this corn lignin which has been de-esterified by treatment with 2M NaOH. Equally important is the absence of peaks correlating carbons at ca. 74-77 ppm with protons at 6.0-6.2 ppm. It is in this region that α-acetylated lignins would give correlations as shown in the synthetic ester models **3*t*** and **3*e*** (Figure 1). A stacked plot (Figure 2) of this same region illustrates that no α-resonances appear above the baseline in the spectrum. Consequently, we can conclusively state that esterification is entirely at the γ-position and not at α.

Long-range C-H correlation (HMBC) spectra, in which a proton will correlate with carbons over 2 or 3 bonds, are ideal for showing connectivity. The carbonyl carbon (C-9) in the spectrum (Figure 3) clearly correlates, not only with H-7 and H-8 protons in *p*CA (hardly informative since that simply implies that the C-9 carbon in *p*CA is within 2 or 3 bonds of the H-7 or H-8 protons in *p*-coumaric acid!), but more importantly with the γ-protons on lignin (peaks at ca. 4.0-4.8 ppm on the proton scale). Unequivocally, *p*CA is attached to the γ-position of lignin. This HMBC experiment, in conjunction with the previous HMQC experiment, conclusively demonstrates that *p*CA is attached exclusively at the γ-position.

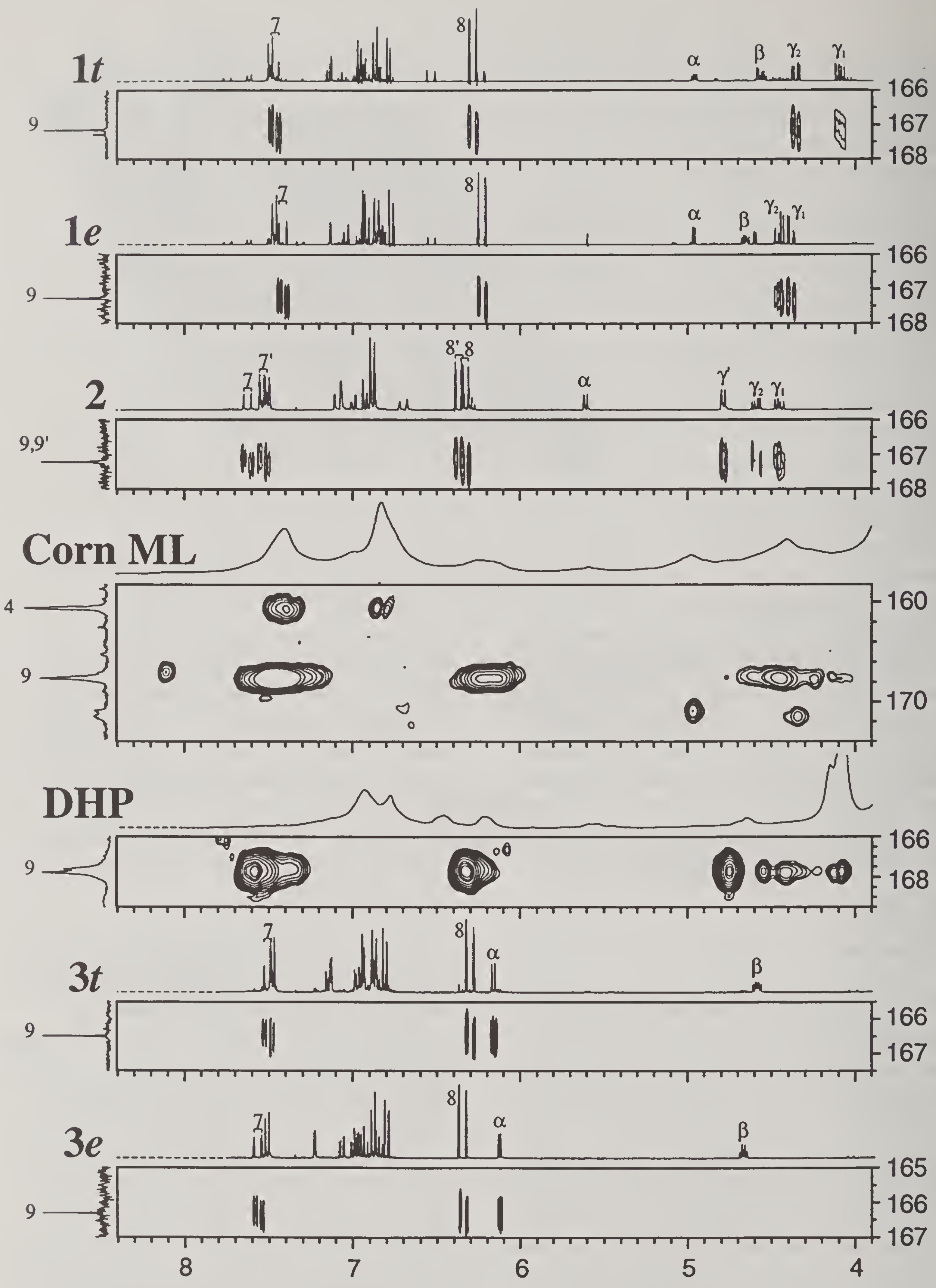
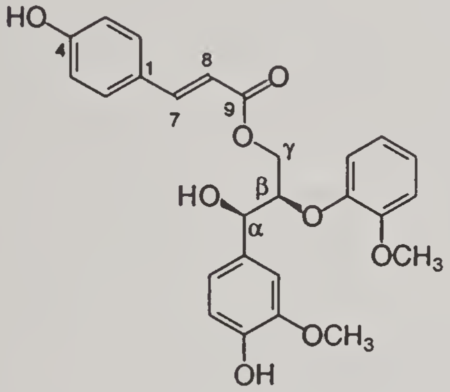
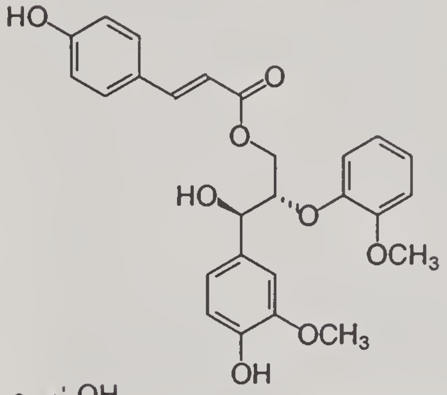


Figure 3. HMBC (long-range C-H correlation) spectrum of corn lignin along with appropriate sections for γ-esterified model compounds ***1*** *and* ***2****, a very low molecular weight DHP containing a substantial proportion of end-groups, and α-esterified compounds* ***3****.*

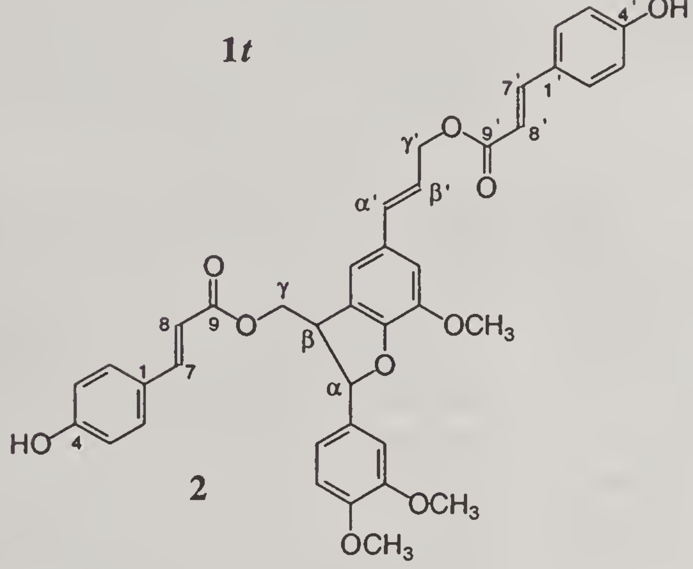
The implication is that single strategy has been used for incorporating *p*CA into the corn lignin structure. That strategy involves the pre-esterification of lignin monomers followed by co-polymerization into the normal lignin polymer. Further implications of this observation are the subject of current work. One completely unresolved and puzzling question is why the plant incorporates so much *p*CA into the lignin — as seen in the following report, this lignin is at least 22% *p*CA implying that this lignin has incorporated some 40+% of these pre-esterified hydroxycinnamyl alcohols.


HO
4
1
8
7
9
O
γ
β
HO
α
O
OCH3
OCH3
OH

1*t*


HO
O
O
HO
O
OCH3
OCH3
OH

1*e*


4'
OH
7'
1'
γ'
O
9'
8'
α'
β'
O
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γ
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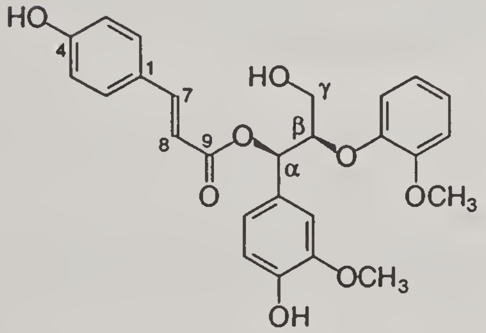
OCH3
7
1
O
α
HO
4
OCH3
OCH3

2


HO
4
1
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HO
γ
β
8
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O
α
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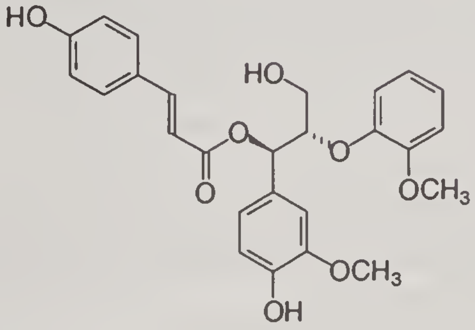
O
OCH3
OCH3
OH

3*t*


HO
HO
O
O
O
OCH3
OCH3
OH

3*e*

*Figure 4: Structures **1** (β-ether-γ-p-coumaroyl ester), **2** (phenylcoumaran-γ-p-coumaroyl ester and coniferyl alcohol-p-coumaroyl ester endgroup, and **3** (β-ether-α-p-coumaroyl ester), used for assignment authentication.*

ISOLATION PROCEDURE FOR CORN LIGNIN FRACTIONS

R.D. HATFIELD, J. RALPH and J. GRABBER

Ronald D. Hatfield

Introduction

Our goal is to elucidate the roles of hydroxycinnamic acids in wall structure which requires an unambiguous assignment of linkage patterns to other wall components. A unique feature of grass lignins is the substantial amounts of esterified *p*-coumaric acid (*p*-CA). A cell wall sample containing a high proportion of esterified *p*-coumaric acid was required for initial studies (see previous report). Mature corn stems were selected for this study based upon screening for *p*-CA using pyrolysis-GC-MS. Isolation of the lignin fraction, relatively depleted in carbohydrates but with the sensitive esters still intact, and solubilization into satisfactory solvents are prerequisites for the detailed regiochemical analysis using NMR spectroscopy.

Methods

The corn lignin fractions were isolated according to the fractionation scheme, in Figure 1. The procedures are relatively straightforward and will not be described in greater detail here.

Discussion

Following the procedures outlined in the fractionation scheme, Cellulysin treatment of ball milled cell walls (CW) resulted in a 65% reduction in total carbohydrate (Fig. 1). This fraction was further separated into D-LPPC and I-LPPC fractions. Compositional analysis of these fractions is compared to the initial CW material in Table 1. Fraction D-LPPC contained approximately 80% lignin and only 15% carbohydrate that was composed primarily of xylose. Fraction I-LPPC on the other hand was rich in carbohydrate (52%) that was composed primarily of glucose. Both fractions contained large amounts of *p*-CA indicating that the fractionation scheme did not result in a partitioning of wall bound *p*-CA.

Of note is the necessity for hot water extraction of the lyophilized dioxane:water soluble fraction, (Fig. 1); for some largely inexplicable reason, the dioxane:water lignin-solution process frees trapped glucose which is not washed out prior to this step. The soluble fraction (S-LPPC) was almost pure monomeric glucose as determined by NMR and carbohydrate analysis and contained no phenolic material. This faction was not subjected to further analysis.

An extremely high amount of dioxane:water soluble lignin (D-LPPC) was obtained using this fractionation procedure (Fig. 1). In typical woody plant materials, ca. 10-20% of the lignin would be expected to be solubilized in this step — here we are seeing some 45% and this material is fully and readily soluble in the NMR solvent system of choice, 9:1 acetone-d_6:D_2O. This is particularly helpful since we wished to analyze a significant and representative fraction of the lignin-*p*-coumaric acid complex.

The I-LPPC fraction is not readily soluble in typical NMR solvent systems and has not been subjected to NMR analysis. Alternative methods of solubilization and/or fractionation of I-LPPC is currently under investigation.

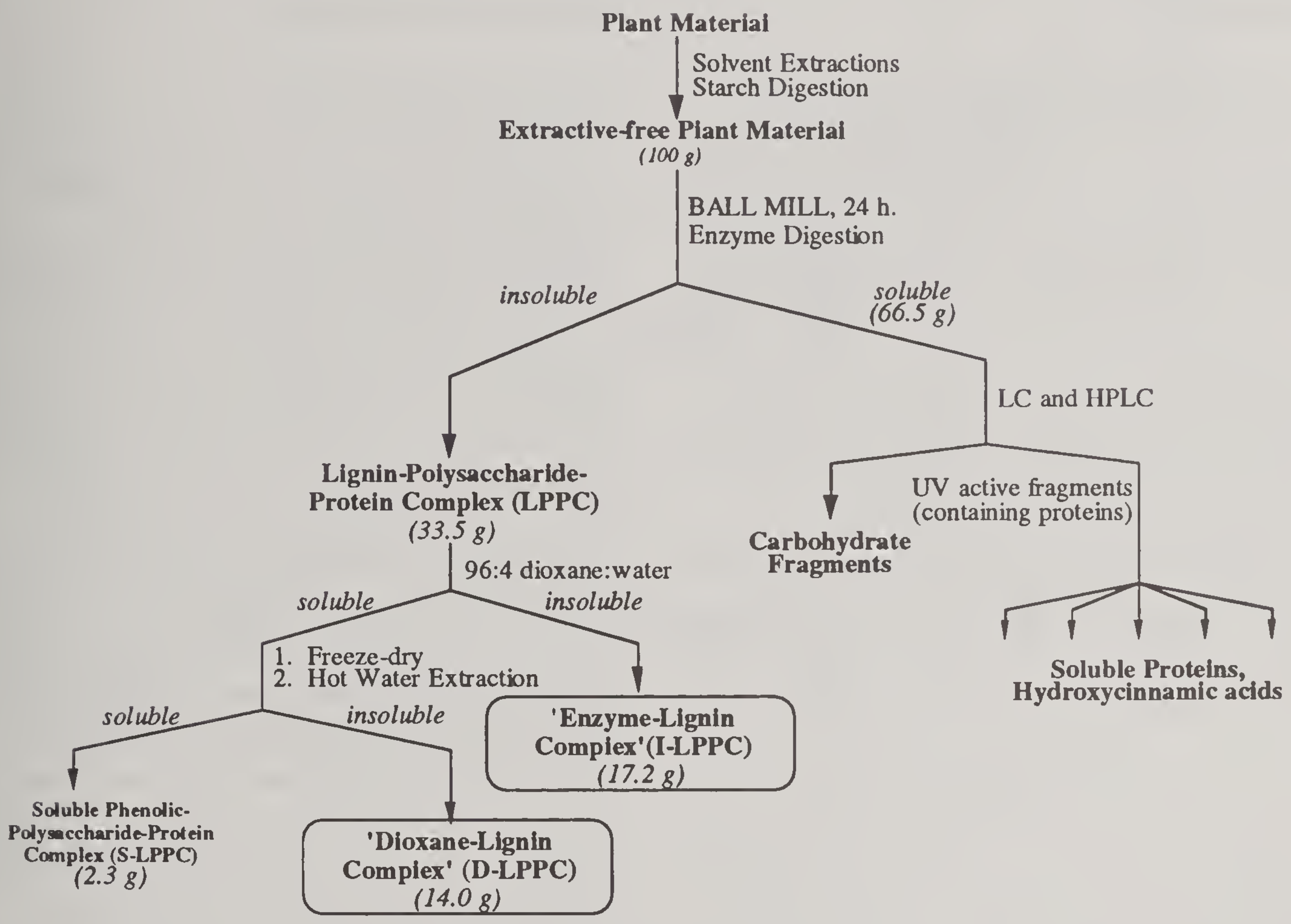

Plant Material
Solvent Extractions
Starch Digestion
Extractive-free Plant Material
(100 g)
BALL MILL, 24 h.
Enzyme Digestion
insoluble
soluble
(66.5 g)
LC and HPLC
Lignin-Polysaccharide-Protein Complex (LPPC)
(33.5 g)
UV active fragments (containing proteins)
Carbohydrate Fragments
96:4 dioxane:water
soluble
insoluble
Soluble Proteins, Hydroxycinnamic acids
1. Freeze-dry
2. Hot Water Extraction
soluble
insoluble
'Enzyme-Lignin Complex'(I-LPPC)
(17.2 g)
Soluble Phenolic-Polysaccharide-Protein Complex (S-LPPC)
(2.3 g)
'Dioxane-Lignin Complex' (D-LPPC)
(14.0 g)

Figure 1. Isolation scheme for lignin-hydroxycinnamic acid fractions from corn. Weights of fractions isolated are in parentheses. The D-LPPC fraction (45% of the corn cell wall) is of intermediate molecular weight and dissolves in 9:1 acetone-d_6:D_2O for NMR. The I-LPPC fraction is of higher molecular weight and is the subject of current investigations.

Table 1. Chemical characteristics of corn rind cell walls and isolated lignin fractions.

	% of Wall fraction									
					Neutral sugars					
Cell Wall Fraction	Klason Lignin	Ferulic Acid	*p*-Coumaric Acid	Total Uronics	Rha	Ara	Xyl	Man	Gal	Glc
CW	20.6	0.55	3.67	2.2	0.21	1.22	21.53	0.15	0.31	43.75
D-LPPC	80.8	0.51	17.40	1.2	0.0	1.56	10.71	0.03	0.10	1.66
I-LPPC	35.7	0.24	4.76	1.7	0.12	0.86	7.2	0.37	0.37	41.51

SYNTHESIS OF GUAIACYL MONOEPOXYLIGNANOLIDES AND DETERMINATION OF THEIR RELATIVE CONFIGURATION VIA LONG-RANGE PROTON COUPLING

S. QUIDEAU and J. RALPH

Introduction

Our current research interests concern the characterization of *p*-hydroxycinnamic acid/lignin complexes in forage plant cell walls. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers led to the identification of new structures which model the possible free-radical coupling products resulting from the involvement of feruloyl esters in the peroxidase-catalyzed lignification processes. Among these structures, we observed a 2,6-diaryl-3,7-dioxabicyclo [3.3.0]octan-8-one system, which arises from a β-β radical coupling between dehydrogenated feruloyl ester and coniferyl alcohol moieties.

The 2,6-diaryl-3,7-dioxabicyclo [3.3.0] octan-8-ones or "2,6-diaryl monoepoxylignanolides" belong to a major group of the naturally occurring lignans. Our first approach toward the synthesis of the desired 2,6-diaryl monoepoxylignanolide, needed for authentication of the structure observed in the dehydrogenation polymer, was the mixed oxidative coupling between ferulic acid and coniferyl alcohol (see the 1991 Research Summaries). This expedient, but low-yielding method gave the expected monoepoxylignanolide, "(±)-MEL" (2,6-exo-bis (4-hydroxy-3-methoxyphenyl)-3,7-dioxabicyclo [3.3.0]octan-8-one) **5**, whose NMR spectroscopic data were in excellent agreement with those of the structure observed in the dehydrogenation polymer. The sustained phytochemical interest in this lignan lactone, a natural germination inhibitor, led us to contemplate an improved synthesis of **5**.

Results and Discussion

A short synthesis of the monoepoxylignanolide "(±)-MEL" (**5**) was accomplished via a regioselective hydride reduction of the dilactone parent **1** (2,6-exo-bis(4-hydroxy-3-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octan-4,8-dione), followed by deoxygenation of the lactone-lactol intermediate *via* silane hydride transfer. The 2-*exo*,6-*endo* (*iso*-MEL) and the 2-*endo*,6-*exo* ("*epi*-MEL") isomers **6** and **7**, and a fourth related compound **8**, featuring a 3-fused 5-membered ring skeleton were additionally formed. 2D phase-sensitive NOESY experiments were used to determine through-space connectivities, and examination of the long range coupled proton network by delayed COSY experiments allowed determination of the relative configuration of these 2,6-diaryl-3,7-dioxabicyclo[3.3.0]octan-8-one structures. As an example, the 2D phase sensitive NOESY spectrum of (±)-MEL (5) (Figure 1, top left) showed strong correlations between H-1 and H-5, and between H-5 and H-4*exo*. H-4*endo* gave a strong response with H-6, proving the *endo*-orientation of the latter. Weaker but still substantial NOE cross-peaks were observed for H-5/H-4*endo*, H-5/H-6 and H-1/H-2, making these NOE data difficult to interpret for configurational determination purposes. The delayed COSY spectrum of **5** revealed a four-bond coupling between the bridgehead proton H-5 and H-2*endo* (Figure 1, bottom right). H-2 was coupled with H-4*exo*, but no coupling was observed with H-4*endo*. Similarly, H-1 correlated with H-4*endo*, but not with H-4*exo*. A weaker cross-peak was observed for H-1/H-6. These

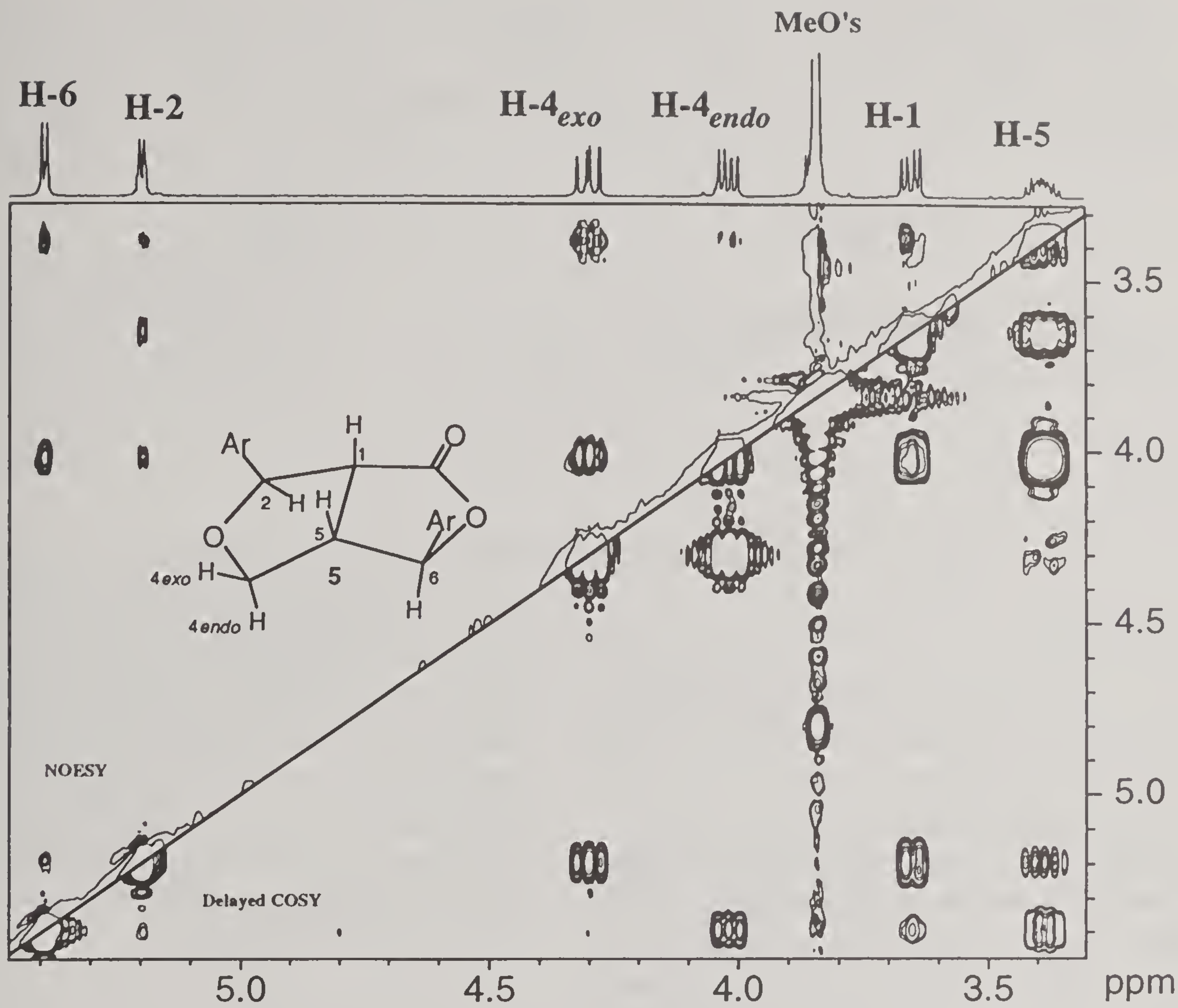
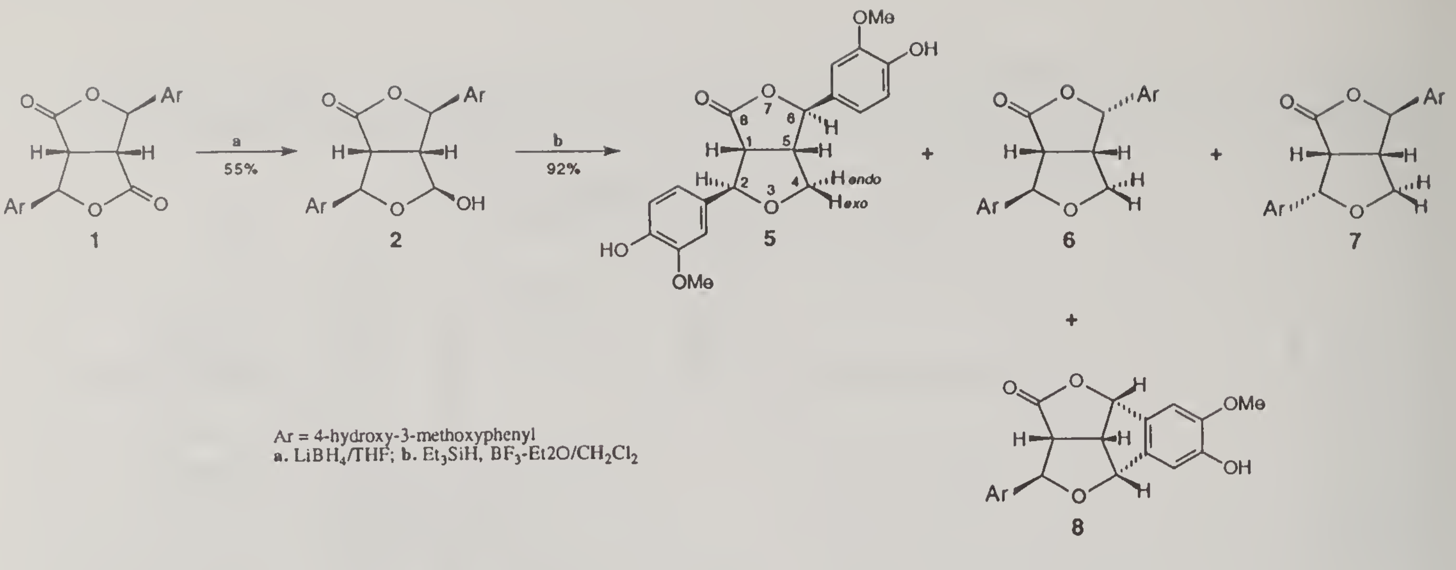


Figure 1. 2D NOESY (top) and 2D delayed COSY spectra of (±)-MEL showing correlations described in text.

^{4}J couplings follow the same type of *transoid* pathway in which an *endo* proton is coupled with a bridgehead proton and with an *exo* proton, allowing the unambiguous establishment of the relative configuration of the molecule. The only cis interaction that gives rise to an observable ^{4}J coupling in **5** is the 1,3-diaxial interaction between H-4*endo* and H-6, which follows an almost planar pathway.

Method

Treatment of **1** with 1.5 equivalents of $LiBH_4$ at ambient temperature in anhydrous THF afforded the required hemiacetal **2**. The lactol **2** was then directly deoxygenated by triethylsilane in the presence of boron trifluoride etherate to afford (±)-MEL (**5**) as the major product, together with its isomers **6** and 7, and compound **8**. The relative proportions were determined from ^{1}H NMR analysis of the reaction product which was subsequently purified by HPLC. The delayed COSY spectrum in Fig. 2 was run with Bruker's cosylr pulse program, using a delay of 250 ms, and the phase sensitive NOESY spectrum was run with Bruker's standard noesytp pulse program, using a mixing time of 1s and a relaxation delay of 4s.



Ar = 4-hydroxy-3-methoxyphenyl
a. LiBH$_4$/THF; b. Et$_3$SiH, BF$_3$-Et2O/CH$_2$Cl$_2$

Figure 2. Synthetic scheme for guaiacyl monoepoxylignanolides 5-8.

Conclusion

Guaiacyl monoepoxylignanolides, including the germination inhibitor (±)-MEL, have been synthesized *via* a selective two-step hydride-silane reduction procedure. The long-range proton coupling network of these 2,6-diaryl-3,7-dioxabicyclo[3.3.0]octan-8-one structures was examined by application of the delayed COSY experiment. The most prominent long-range couplings occurred between *trans*-interacting protons, e.g. a benzylic proton having an *endo* orientation exhibits a readily detectable ^{4}J coupling with a bridgehead proton. These observations allowed determination of the relative configuration of the prepared compounds, making the delayed COSY experiment an efficient alternative to the use of NOE spectroscopy.

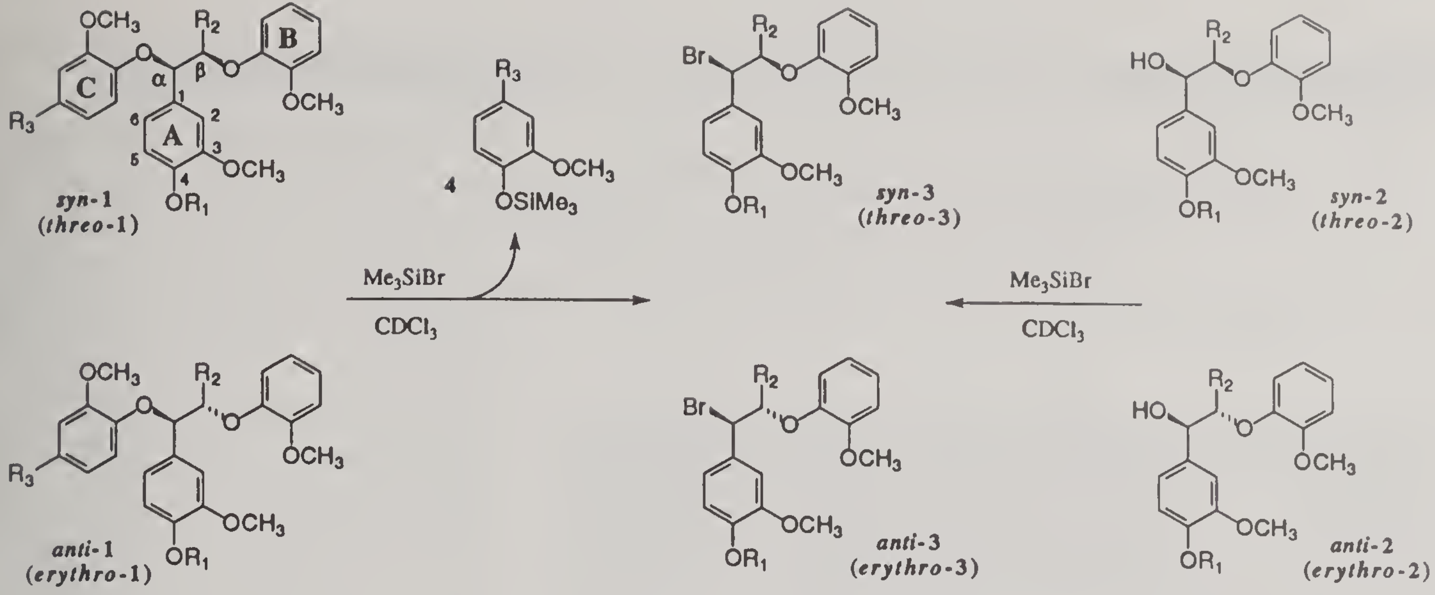
STEREOSELECTIVITY IN BENZYL ARYL ETHER CLEAVAGE BY BROMOTRIMETHYLSILANE

J. RALPH and R.F. HELM

Introduction

The natural complexity of lignin provides a challenge to chemists interested in the quantitative aspects of lignin structure and the chemical reactivity of specific subunits. A common approach to obtain this information is via the synthesis of lignin model compounds which are used as the basis of chemical and spectroscopic studies. Our investigations into the structure and cleavage of benzyl aryl ether (**1**) and benzyl alcohol (**2**) lignin models (Figure 1) have revealed an interesting diastereoselectivity exhibited by bromotrimethylsilane (TMSBr) for the α-ether cleavage of compounds **1** as well as for the bromination of benzylic alcohols **2**.

The bromination of benzyl alcohols (including lignin models **2**) by TMSBr is a facile route to benzyl bromides. Treatment of 4-hydroxybenzyl alcohols such as **2a** with TMSBr provides a convenient preparation of important quinone methide intermediates for lignin investigations. We were interested in developing a selective benzyl aryl ether cleavage method for α-etherified lignin model compounds such as **1a** and **1e**, and the use of TMSBr was therefore explored.



Cmpd	R_1	R_2	R_3
1a	H	CH_2OH	$CH=CH\text{-}COOCH_3$
1b	CH_3	CH_2OH	$CH=CH\text{-}COOCH_3$
1c	Ac	CH_2OAc	$CH=CH\text{-}COOCH_3$
1d	CH_3	CH_2OAc	$CH=CH\text{-}COOCH_3$
1e	H	CH_2OH	CH_2OH
1f	CH_3	CH_2OH	CH_2OH
1g	CH_3	CH_2OAc	CH_2OAc
2a,3a	H	CH_2OH	
2b,3b	CH_3	CH_2OH	
2c,3c	CH_3	CH_2OAc	
2d,3d	CH_3	CH_3	
4a			$CH=CH\text{-}COOCH_3$
4b			$CHBr\text{-}CH_2\text{-}COOCH_3$
4c			CH_2OH
4d			CH_2OAc
4e			CH_2Br

*Figure 1. Action of TMSBr on benzyl ethers **1** and benzyl alcohols **2**.*

Methods

Bromination/Ether Cleavage: Benzyl aryl ethers **1** or benzyl alcohols **2** (2-5 mg) were dissolved in CDCl3 (400 μl) in a 5 mm NMR tube. TMSBr (1-2 μl, ca. 2 eq) was added and the tube shaken and transferred to the NMR probe at 300 K within 1 min. An initial spectrum (t = 2-3 min after TMSBr addition) was taken and further spectra acquired at selected intervals. Bromides **3** from ethers **1** were spectroscopically identical to those produced by bromination of alcohols **2**. Isomer ratios were measured from integration of the α-proton doublets.

Discussion

NMR-tube α-aryl ether cleavage of models **1a** and **1e** (Figures 1 and 2) was extremely rapid and selective; the β-aryl ether linkage remained intact over a period of days at room temperature. We planned to gain some further insight into the stereochemistry of these α-aryl ethers, since the *threo/erythro* assignment is currently based on chemical shifts of the γ-protons and on the *erythro*-selectivity of addition of phenols to quinone methides. However, the cleavage reaction of free-phenolic benzyl aryl ethers **1a** and **1e** was too fast at room temperature and stereochemical scrambling occurred before NMR measurements could be completed.

We had previously noted slow scrambling of the α-bromide of *erythro*-**2d** which possessed a 4-methoxyl rather than a 4-hydroxyl substituent in ring A. Thus methylation of **1a** and **1e** to compounds **1b** and **1f** and subsequent exposure to TMSBr provided α-bromides **3b** which also showed an improved stability. Further improvement in bromide stability was obtained by acetylation of the remaining hydroxyl group (compounds **1d** and **1g**) – we assume that the isomerization is aided by the

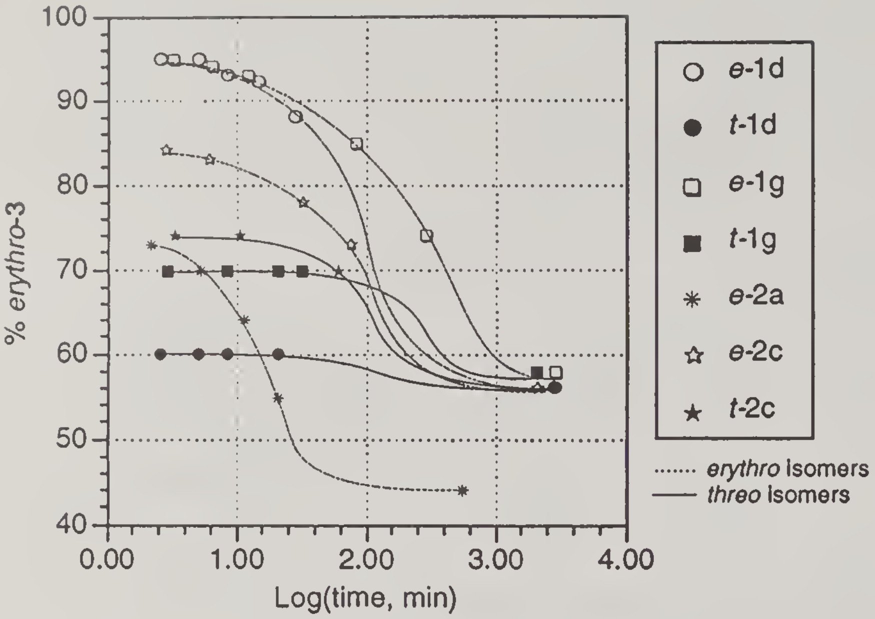


*Figure 2. Bromination of alcohols **2** or cleavage of ethers **1** gives bromides **3** which isomerize. Percent erythro-**3** is plotted against time (on a log scale) showing the stereoselectivity of the reaction (short time) and the isomerization rate and final equilibrium rations of bromide **3**.*

presence of HBr. Benzyl-aryl ether cleavage of the phenolic acetate **1c** was not detectable in the course of a two day exposure to TMSBr at room temperature. This indicates that the benzyl ring must be electron rich for the benzyl aryl ether cleavage reaction to occur under these conditions. Using 2-5 mg of compound and ca. 2 eq of TMSBr in 400 µl $CDCl_3$ in an NMR tube, ethers *erythro*-**1d** and *erythro*-**1g** (Figure 2) were completely cleaved to bromides **3c** within 10 min at 300 K, whereas *threo* ethers *threo*-**1d** and *threo*-**1g** required 12 and 30 min respectively for complete conversion. Isomerization of bromides **3c** to the thermodynamic equilibrium mixture was complete in 24 h, giving 56 ± 2:44 ± 2 *erythro:threo* **3c** in all cases (Figure 3). Bromides **3a** (derived from models **1a**, **1e**, or **2a**) equilibrated to a 44:56 *erythro:threo* mixture.

The plot in Figure 2 shows the initial bromide *erythro:threo* ratios and indicates the equilibration rates of the bromides **3**. As noted previously, stereochemical assignments of bromides **3** are based on γ-proton chemical shifts. It is clear that bromination of *erythro*-**2c** and, more dramatically, cleavage of *erythro* ethers of **1d** or **1g** yield bromides **3c** with *high retention of configuration*. The *threo* isomers of **1d** and **1g**, however, undergo predominantly an inversion of configuration but with a significantly lower degree of selectivity. Finally, bromination of the less-reactive γ-acetate *erythro*-**2c** is more diastereoselective than *erythro*-**2a**, and the equilibration of bromides **3c** is slower than **3a**. The similar behavior of *erythro*-**2c** and the *erythro* ethers of **1d** and **1g** in giving retention of configuration is consistent with the stereochemical assignments. Thus the *erythro* benzyl aryl ethers appear to cleave and brominate by a concerted mechanism whereas the *threo* isomers undergo a more complicated bromination process which may include both concerted and non-concerted pathways.

In summary, lignin model benzyl aryl ethers are cleaved cleanly and selectively with TMSBr, *erythro* isomers producing *erythro* bromides with high (ca. 95%) diastereoselectivity.

FORAGE QUALITY CHARACTERISTICS OF MULTIFOLIOLATE ALFALFA

D.R. BUXTON, I.T. CARLSON, S.K. BARNHART and K.G. BIDNE

Dwayne R. Buxton

Alfalfa leaves decrease in forage quality more slowly than stems with advancing maturity and leaves have higher nutritive value and intake potential than stems when alfalfa is normally harvested. Therefore, forage quality of alfalfa should improve as the leaf/stem ratio increases through genetic modifications. The multifoliolate leaf trait of alfalfa, which results in leaves with more than three leaflets per leaf, may be one method of developing plants with higher leaf/stem ratios. Generally, individual leaflets of multifoliate plants weigh less than those of trifoliates, but composite leaf weight is greater in multifoliates. Multifoliolate plants may have fewer leaves per stem, however.

Although several studies have shown increased leaf/stem ratios in multifoliolate alfalfa compared to trifoliolate alfalfa, little information is available on the influence of the multifoliolate leaf trait on herbage quality even though alfalfas with the multifoliolate trait are now marketed by several companies. This study was conducted to compare morphological and forage quality characteristics of three multifoliolate cultivars with three standard cultivars. Forage quality was determined by measuring neutral detergent fiber (NDF) concentration, a measure of intake potential of forages, acid detergent fiber (ADF) concentration, often use as an estimate of forage digestibility, and crude protein (CP) concentration.

Materials and Methods

Observations were made on four field replicates of ‘MultiKing 1’, ‘Legend’, and ‘MultiPlier’ multifoliolate alfalfas and on ‘5262’, ‘Dart’, and ‘Vernal’ trifoliolate alfalfas in an alfalfa variety trial at Ames, IA. Morphological characteristics and forage quality were determined only on the first two harvests, but yield was determined on four harvests. Morphological measurements included maturity by the mean stage count by the Kalu and Fick method (2 = late vegetative, 3 = early bud, 4 = late bud), number of main stem nodes without attached leaves, number of leaflets per main stem leaf, and leaf/stem mass ratio.

Additionally, NDF, ADF, and CP concentrations were determined for the total herbage.

Results and Discussion

The multifoliolate characteristic was expressed more strongly in the spring than in the summer with an average of 3.45 leaflets per leaf on May 31 and 3.21 on July 6 (Table 1). The number of leaflets per leaf was significantly greater for the multifoliolate cultivars than for the trifoliolate cultivars at both harvests. MultiKing 1 had the strongest expression of the multifoliolate characteristic, especially in the summer.

In the spring, the multifoliolate cultivars had a similar leaf/stem ratio that averaged 0.73, compared to 0.63 for the trifoliolate cultivars. This difference was reflected in a lower ADF concentration in the multifoliolate cultivars (31.4%) compared to the trifoliolate cultivars (33.0%) and in a lower NDF concentration in the multifoliolate cultivars (41.2%) compared to the trifoliolate cultivars (42.5%) (Table 2). The higher leaf/stem ratio of the multifoliolate cultivars did not result in higher CP concentrations, which may be because of lower CP concentrations in the leaf fractions of multifoliolate cultivars. Weak expression of the multifoliolate characteristic during the July harvest, especially by Legend and Multiplier, resulted in no difference in leaf/stem ratio or in ADF, NDF, or CP concentrations. Small differences in maturity occurred in the spring, but there were no maturity differences in the summer (Table 1). Furthermore, there were no differences in the number of nodes without leaves among the cultivars, indicating that there was no difference among the cultivars in leaf loss.

Yield of the multifoliolate cultivars was similar to the trifoliolate cultivars (Table 1). Total yield of the two harvests during the establishment year (the year before this study was conducted) averaged 3.17 tons/acre for the multifoliolate cultivars and 3.18 tons/acre for the trifoliolate cultivars. Total yield of the four harvests during the year of this study was 7.05 tons/acre for the multifoliolate cultivars and 6.98 tons/acre for the trifoliolate cultivars.

This study shows that the multifoliolate characteristic does not always result in higher forage quality. Cultivars that have consistently high expression of this trait may have higher digestibility and intake potential than normal cultivars, but probably will not have higher CP concentrations. Cultivars with this trait do not seem to have reduced yield potential.

Table 1. Dry matter yield, stage of development, and morphology of three multifoliolate alfalfa cultivars compare with three trifoliolate cultivars.

	May 31			July 6		
Cultivar	Yield	Maturity	Leaflets/ leaf	Yield	Maturity	Leaflets/ leaf
	t/ac			t/ac		
Multifoliolate						
MultiKing 1	2.50	2.8	3.58	1.86	4.1	3.45
Legend	2.63	2.7	3.44	1.76	4.0	3.08
Multiplier	2.73	2.7	3.32	1.78	4.1	3.10
Trifoliolate						
5262	2.68	2.3	3.02	1.87	3.9	3.00
Dart	2.77	2.7	3.00	1.88	4.0	3.01
Vernal	2.63	2.6	3.01	1.49	3.9	2.98
LSD (0.05)	0.15	0.2	0.14	0.17	NS	0.18

Table 2. Quality characteristics of three multifoliolate alfalfa cultivars compared with three trifoliate cultivars.

	May 31			July 6		
Cultivar	NDF	ADF	CP	NDF	ADF	CP
	%					
Multifoliolate						
MultiKing 1	40.8	30.3	21.8	45.6	31.6	20.2
Legend	41.3	31.6	22.6	44.6	29.5	21.8
Multiplier	41.5	32.6	22.8	44.0	30.7	21.6
Trifoliolate						
5262	42.4	33.0	21.3	44.7	29.6	19.6
Dart	42.5	33.0	22.2	45.8	31.6	19.5
Vernal	42.6	33.0	21.6	45.8	30.1	19.3
LSD (0.05)	1.46	NS	NS	NS	NS	NS

GENETIC VARIATION AMONG MAIZE INBREDS FOR CELL-WALL COMPONENTS AND DIGESTIBILITY

J.P. LUNDVALL, D.R. BUXTON, A.R. HALLAUER and J.R. GEORGE

Introduction

Forage maize (*Zea mays*, L.), conserved as whole-plant silage, is a high-energy component of dairy cow rations. Grain supplies much of the energy, but approximately half of the harvested organic matter is stover (aboveground vegetation less ears and grain). The grain portion of forage maize is highly digestible; however, stover cell-wall components—especially lignin—limit digestibility, energy availability, and overall feeding value of whole-plant silage. Improved maize silage hybrids should be selected for improved stover digestibility rather than simply high grain yield, as has been usually recommended. Little information is available regarding genetic variation for stover cell-wall component concentrations and digestibility among maize inbreds. This study was conducted to 1) determine genetic variation for cell-wall component concentrations and digestibility among a group of maize inbreds; 2) compare inbred cell-wall component concentrations at silking and at physiological maturity; 3) examine the relationship between maize leaf and stem quality. Among the inbreds were four brown-midrib (bm) mutant genotypes. The bm mutation typically affords reduced lignin and cell-wall concentrations compared to normal isolines; however, the mutation also increases lodging susceptibility and decreases yield potential. Normal isolines of the bm inbreds were not included in this study.

Materials and Methods

Fifty maize inbred genotypes were grown in a replicated field trial near Ames, IA in 1989 and 1990. Basal two elongated stem internodes and collared leaf blades were harvested near the silking stage of maturity to evaluate stover quality before ear and grain development. Stem internodes were also harvested at physiological maturity. Sufficient plant material from each of 45 of the inbred genotypes was available for laboratory analysis of cell-wall component concentrations and in vitro digestible dry matter (IVDDM).

Results and Discussion

Highly significant ($p \leq 0.01$) genetic variation was noted among the inbreds for cell-wall component concentrations and IVDDM of stems and leaf blades. Genetic variation for each forage quality measurement was largest in mature stems and smallest in leaf blades (Table 1). Brown-midrib inbreds were similar to other inbreds for cell-wall component concentrations and IVDDM. Stem neutral detergent fiber (NDF) concentration was negatively correlated with IVDDM near silking ($r = -0.74$) and at physiological maturity ($r = -0.82$). Stem lignin (ADL) concentration was negatively correlated with IVDDM near silking ($r = -0.62$) and at physiological maturity ($r = -0.79$). Leaf IVDDM was negatively correlated with concentrations of NDF (- 0.31) and ADL ($r = -0.61$) near silking. Stem IVDDM near silking was not closely related to stem IVDDM at physiological maturity ($r = 0.40$) nor to leaf IVDDM ($r = 0.31$). Stem ADL concentration near silking was not closely related to stem ADL at physiological maturity ($r = 0.41$) nor to leaf ADL ($r = 0.24$). Stem NDF concentration near silking was not closely related to stem NDF at physiological maturity ($r = 0.10$) nor to leaf NDF ($r = 0.14$).

Conclusions

The results indicate that highly significant genetic variation exists for cell-wall component concentrations and IVDDM among maize inbreds without use of the bm trait. Discovery of inbred variation for stover quality offers the potential to develop silage hybrids of maize with improved cell-wall utilization by dairy cows. However, it may not be possible to predict stem forage quality at normal silage harvest maturity based on samples collected near silking. Likewise, sampling in breeding programs should probably be based on total stover and not on plant parts.

Table 1. Genetic variation among 45 maize inbreds sampled near silking and at physiological maturity for cell-wall component concentrations and in vitro digestibility.

Quality Measurement	Minimum	Mean	Maximum	$LSD_{0.05}$[a]
Immature stems	- - - - - - - - - - - -	g kg^{-1} dry matter	- - - - - - - - -	
NDF	489	584	665	19
ADL	30	42	57	3
Acid Detergent Fiber (ADF)	312	380	457	16
IVDDM	465	622	727	31
Leaf blades				
NDF	517	569	602	16
ADL	26	41	56	5
ADF	274	302	323	12
IVDDM	580	630	676	20
Mature stems				
NDF	510	679	791	37
ADL	40	58	89	5
ADF	353	454	570	26
IVDDM	262	470	650	49

[a] LSD used to separate individual inbreds.

MAIZE INTERNODE ELONGATION PATTERNS

T.A. MORRISON, J.R. KESSLER and D. R. BUXTON

Introduction

Preliminary information on the rates and patterns of elongation for individual maize internodes was necessary to conduct detailed studies on the process of cell-wall lignification. This report summarizes photo-documentation of maize stalk elongation.

Materials and Methods

Maize seedlings of single-cross hybrid Mo17 ´ B73 were grown in a growth chamber. When internode 7 (I7), the 2nd internode above the soil line, was ~10-mm long, it was dotted along its length with black acrylic paint at 1-mm intervals and photographed every 24 h. Full-scale photographs documented daily incremental increase in elongation of I7. Internodes 8 through 15 were also dotted and photographed in the same manner as they reached 10-mm length. Following an analysis of variance for a split-split plot design, with internode as the main plot, dot location as the sub-plot, and day as the sub-sub-plot, the sum of squares for internode was subdivided using single degree of freedom linear comparisons. From this analysis, internodes were divided into statistically similar groups.

Results and Discussion

In general, elongation of each above-ground internode of maize followed a basipetal wave pattern (Fig. 1). Elongation began uniformly throughout the internode for the first 2-3 days, then shifted to the upper region of the internode. Elongation shifted basipetally from the upper region toward the middle, and later toward the basal region of the developing internodes. The most rapid elongation (growth rate) occurred in the basal few centimeters of each internode in the last few days of their elongation periods. Variations in final internode length and length of the elongation period were factors that distinguished the groups of similarly-behaving internodes. Fig. 2 shows the groupings of internodes having similar growth rates based on single degree of freedom linear comparisons. Elongation patterns for each group were not drawn for lack of space. Fig. 1 shows the general elongation pattern without the variations that distinguished the internode groupings.

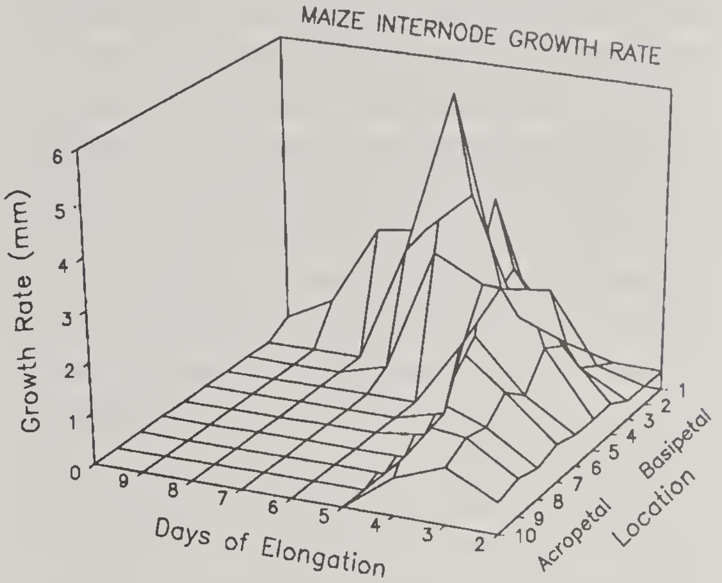


Figure 1. General elongation pattern for a maize internode.

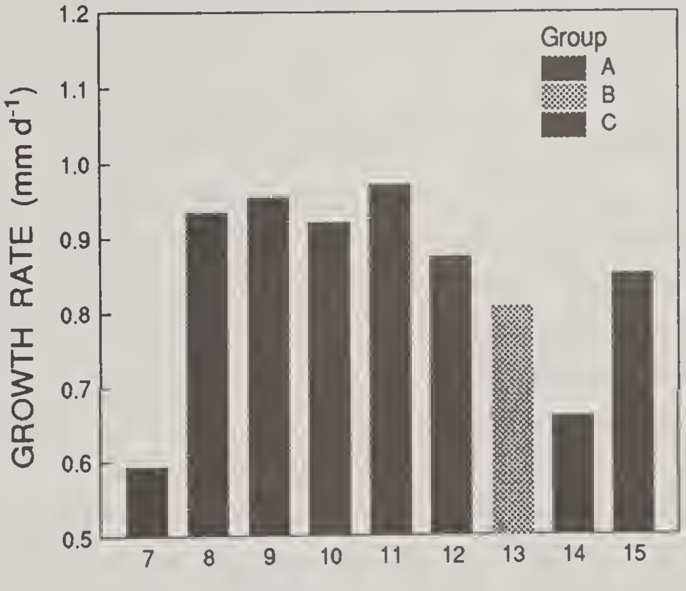


Figure 2. Groupings of internodes having similar growth rates.

Internodes 7 (I7) and 14 (I14) show similar, truncated growth rates due to residual root characteristics (I7) and reduced leaf carbohydrate supply (I14). Carbohydrate from the leaf attached at the base of I14 may have been diverted to I13 and its developing ear shoot, and carbohydrate supply from the leaf at node 15 may have been reduced due to the smaller final size of maize upper leaves. Internodes 8 through 12 and I15 had similar growth rates. These internodes were vegetative with no residual root characteristics or reproductive ear shoot influences. I8 - 12 elongated fully and had the greatest daily growth rate (Fig. 2). I15 elongated fully (as did I16 - I18) in order to elevate the terminating tassel. Internode 13 showed slower growth rate; probably it was in competition with its associated developing ear shoot for leaf carbohydrate.

The period of time required for each internode to fully elongate increased acropetally from I7 through I13, then decreased for I14 through I18. The longer the final length of internode the longer the elongation period required. Also, the developing ear appeared to delay cessation of elongation.

Conclusion

Knowing the elongation patterns for given maize internodes may be an aid to crop physiologists working on more detailed studies with maize.

GENETIC VARIATION AMONG INBRED MAIZE LINES: CELL-WALL CONCENTRATION, COMPOSITION AND DEGRADABILITY

H. G. JUNG and D. R. BUXTON

Introduction

Maize silage is a major feed resource for dairy production systems. Approximately half of the harvested organic matter consists of the stover fraction of the plant. While the grain in maize silage is highly digestible, the high cell-wall concentration of the stover fraction limits its utilization by dairy cattle. The usual recommendation to farmers in selecting a maize hybrid for use as silage is to choose a high grain-yielding variety. However, as we consider options for improvement of maize as a silage crop, selection for increased grain content may not be the best strategy for numerous reasons. High levels of grain in the diet can result in milk-fat depression and acidosis. Reduced stover cell-wall concentration may result in poor agronomic performance of maize such as increased lodging and stress susceptibility. A more viable alternative for improvement of maize as a silage crop would be to increase the utilization of the stover cell walls by dairy cattle through selection of maize for increased cell-wall degradability. Our objective in this study was to examine a group of inbred maize lines for genetic variation in cell-wall concentration, composition and degradability.

Materials and Methods

Fifty inbred maize lines were grown in a replicated field trial at Ames, IA in 1989 and 1990. The basal two stem internodes were harvested at the silking stage of maturity. One field replicate from each of 47 of the inbred lines was available from 1989 and 1990 for use in this analysis. Total fiber concentration and composition was determined by acid hydrolysis of starch-free, alcohol insoluble residues. Individual neutral sugars and total uronic acids were determined. The ash-free, non-hydrolyzed residue represented Klason lignin. Ester- and ether-linked *p*-coumaric and ferulic acids of the cell wall also were measured. Maize internode samples were fermented *in vitro* with ruminal fluid for 24- and 96-h to determine the rapidly fermentable polysaccharide fraction and the fraction which was ultimately available for degradation. Individual neutral sugars and total uronic acid degradabilities were calculated.

Results and Discussion

For virtually every cell-wall characteristic examined, significant ($P < 0.05$) genetic variation was found among the 47 inbred maize lines examined. Total fiber concentration of the inbred line with the highest concentration was almost 100% greater than that observed for the lowest line (Table 1). For the major components of the wall, uronic acids and Klason lignin varied over a two-fold range while the neutral sugar component of polysaccharide deviated substantially less. Esterified *p*-coumaric acid concentrations were high as expected and had a 300% range among the lines. Phenolic acids that were ether-linked also varied widely among lines, with undetectable levels for both phenolic acids in some lines. Just as for cell-wall concentration and composition, wall polysaccharide degradability varied widely among inbred lines. The genetic variation for total polysaccharide degradability was greater for 24-h extent of degradation (3x) than after 96-h (1.75x). Arabinose and uronic acids were highly degraded after only 24-h of fermentation and little further degradation of these sugar components was observed with additional fermentation time. In contrast, glucose and xylose degradation was relatively low after 24-h, but increased substantially with further fermentation. Interestingly, at 96-h the extent of xylose degradation did not differ among the lines.

Conclusion

The results indicate very clearly that significant and substantial genetic variation exists among inbred maize lines for most all cell-wall characteristics. Variation is especially great for lignin. The observed variation in wall polysaccharide degradability suggests that it should be possible to develop maize hybrids from these inbreds with improved cell-wall utilization by dairy cows. The obvious next step is to intermate selected inbred lines to determine if the resultant maize hybrids are indeed better for silage production and animal feeding.

Table 1. Data for variation among 47 inbred maize lines for cell-wall concentration, composition and degradability.

Characteristic	Mean	Minimum	Maximum	SD
	----------	g kg^{-1} Organic matter	----------	
Total fiber	655	438	812	69
	----------	g kg^{-1} Total fiber	----------	
Neutral sugars	760	680	840	31
Uronic acids	47	26	71	10
Klason lignin	158	91	227	26
Phenolic esters				
p-Coumaric	24	10	34	5
Ferulic	4	3	7	1
Phenolic ethers				
p-Coumaric	5	0	20	4
Ferulic	3	0	7	1
	----------	g kg^{-1}	----------	
Polysaccharide degradation				
24-h	544	262	857	10
96-h	758	505	897	8

DIVERSITY AMONG MAIZE INBRED LINES FOR IN VITRO DIGESTION KINETICS

R.W. HINTZ, D.R. MERTENS, D.R. BUXTON and H.G. JUNG

Introduction

Utilization of maize silage by dairy cattle could be increased either by decreasing the concentration of fiber in the plant or by increasing the rate or extent of fiber digestion. Genetic variation for fiber concentration has been observed in maize and is used by breeders as a selection criteria for developing new inbred lines; however, no information is currently available on the variation in fiber digestion kinetics. This study was designed to determine if significant variability for fiber digestion kinetics existed among maize inbred lines and to determine the relationship among fiber digestion kinetic parameters.

Materials and Methods

Lower stem internode tissue of 47 maize inbred lines harvested at silking during 1989 and 1990 were analyzed for initial NDF concentration (NDF_o), indigestible NDF residue concentration (INDF) and rate of digestion (k_d) using the in vitro true digestibility procedure of Goering and Van Soest (1970). A 0.2 g sample of each inbred from each year was incubated under constant CO_2 pressure for 11 time periods (0, 3, 6, 12, 18, 24, 36, 48, 72 and 96 h). Neutral detergent fiber residues were determined using 0.5 g of sodium sulfite per sample and adding a heat stable amylase during filtration.

The fiber digestion kinetics model of Mertens (1973) was fitted using nonlinear regression as described by Grant and Mertens (1992).

$$Y = D_o e^{-K_d(t-L)} + INDF$$

where

Y = NDF residue at time t, D_o = potentially digestible NDF (percentage of initial DM), K_d = fractional rate constant of digestion (per hour), L = discrete lag (h), t = time (h), and INDF = indigestible NDF residue (percentage of initial DM).

Results and Discussion

Although statistically significant, the correlations among kinetic parameters were not perfect, indicating that variation for one parameter cannot be entirely explained by changes in other parameters (Table 1). This indicates that it may be necessary for a breeder to select for all characteristics of interest to assure that progress is made in a breeding program. Digestion rate was significantly correlated with NDF_o and INDF, but variation in these fiber concentrations explained only half of the variability observed in rate of digestion. This indicates that the composition of fiber may be as important as its concentration to plant dry matter in determining digestion rate. The correlation between NDF_o and INDF was also less than 1, supporting the hypothesis that the composition of NDF may vary among maize inbred lines and that these compositional differences are important in determining the extent to which NDF is digested. The magnitude of differences observed for in vitro digestion kinetics among inbred lines indicates that there should be sufficient genetic variability to permit breeders to select maize genotypes for specific kinetic characteristics. Statistically significant differences among inbred lines were present for all kinetic parameters (Table 2).

Table 1. Correlation coefficients between in vitro digestion kinetic parameters.

Parameter	INDF	K_d
NDF_o	.70	-0.52
INDF		-0.44

All correlations significant at the 1% probability level.

Table 2. Initial NDF (NDF$_o$), indigestible NDF (INDF) and digestion rate (K$_d$) coefficients for 47 maize inbred lines.

Inbred	NDF$_o$	INDF	K$_d$	Inbred	NDF$_o$	INDF	K$_d$
	(g kg^{-1})	(g kg^{-1})			(g kg^{-1})	(g kg^{-1})	
B14A	636	284	0.0608	bm1bm1	570	182	0.0960
B14A02	681	280	0.0388	bm2bm2	495	200	0.0767
B37	623	227	0.0462	bm4bm4	547	175	0.0603
B3702	600	217	0.0610	BS16VC2	598	265	0.0723
B52	699	290	0.0404	L289	640	259	0.0446
B57	507	139	0.0609	L317	491	209	0.0501
B64	603	257	0.0260	LAN232	571	205	0.0590
B68	647	251	0.0428	LAN496	623	322	0.0509
B73	562	220	0.0698	M017	578	206	0.0718
B7302	583	204	0.0633	N7A	623	198	0.0608
B75	610	286	0.0598	NC252	592	225	0.0719
B76	573	218	0.0613	NC254	552	205	0.0539
B77	533	169	0.0792	NC256	605	269	0.0603
B78	559	207	0.0725	NC258	597	166	0.0640
B79	613	257	0.0622	NC262	582	238	0.0527
B84	590	248	0.0628	NC264	581	204	0.0591
B86	558	240	0.0523	NC266	594	259	0.0763
B87	591	208	0.0702	NC268	556	216	0.0626
B88	638	236	0.0614	NC270	593	242	0.0666
B89	628	230	0.0421	NC272	547	201	0.0616
B90	593	202	0.0485	R225	544	220	0.0830
B91	559	195	0.0605	R226	558	230	0.0501
B93	554	21	0.0563	R227	465	159	0.0933
B94	582	201	0.0592				
LSD (0.05)	61	55	0.0223				

RELATION BETWEEN MONOLIGNOL-SYNTHESIZING ENZYME ACTIVITY AND CELL WALL FIBER COMPONENTS OF MAIZE INTERNODES

T.A. MORRISON and D.R. BUXTON

Introduction

Lignin is a serious limiter of forage digestibility by ruminant animals. Efforts are underway to study the process of lignification with the goal of determining ways to reduce the negative effects of lignin on digestibility. The enzymes of phenylpropanoid and lignin biosynthesis are known as is the sequence in which they act. But there is little information on which enzymes regulate the production of the final lignin product and at what plant physiological stage these pathway enzymes are engaged. Both the key 'lignification' enzymes and the plant physiological stages during which they are active must be determined. The action of the first enzyme of the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL), is necessary for the production of many primary plant metabolites including flavanoids, tannins, phytoalexins, and lignin. PAL's importance as a lignin regulatory enzyme has been questioned. Recent research indicates that the last enzyme of the lignin pathway, cinnamyl alcohol:NADPH dehydrogenase (CAD), may play a major regulatory role in lignin biosynthesis as it acts just prior to random peroxidase-assisted polymerization of cinnamic alcohols to lignin. Both PAL and CAD appear to be necessary for production of lignin, but it is not clear how they actually correlate with cell-wall fiber content.

The objective of this study was to determine the patterns of PAL and CAD activity in internodes of maize exhibiting a range of physiological development stages and to correlate their activities with cell-wall lignin measured as acid detergent lignin (ADL). The activity of tyrosine ammonia-lyase (TAL), which behaves essentially like PAL, and contents of cellulose (CELL) and hemicellulose (HEMI), which behave similarly to ADL, were also measured. Maize stalks at the 14th leaf stage were chosen for study because individual internodes were of different ages and expressed various phases of development: lower internodes were lignifying, middle internodes were actively elongating and beginning to lignify, and the upper internodes were just beginning to elongate. It was anticipated that the relative importance of each enzyme to lignin production could be determined from the correlations.

Materials and Methods

Maize seedlings of single-cross hybrid Mo17 ´ B73 were grown in a growth chamber under 28°/18°C day/night, 12 h photoperiod, and 960 ± 60 mmol m^{-2} s^{-1} photosynthetically active radiation at canopy height. At the 14th leaf stage, Internodes 7 through 14 were excised, divided into thirds, frozen at -20°C, and freeze-dried from 20 plants. Samples from four plants were combined for each internode section, yielding five replicates. Internode section composites were finely ground. For PAL and CAD enzyme assays, 0.5 g samples were placed in an ice water bath and sonicated for 45 min in 7.5 mL of 0.1 M Tris-HCl buffer containing 20 mM 2-mercaptoethanol and 0.5 % poly(ethylene)glycol. For ADL, CELL, and HEMI determinations, 0.5 g samples were assayed by the Goering and Van Soest method. Analysis of variance was used in a split-plot design with internode assigned to the main plot and location within internode to the subplot. CAD activity was determined spectrophotometrically by the Wyrambik and Grisebach assay. PAL activity was determined according to the Abell and Shen assay.

Results and Discussion

PAL activity was found to be associated with tissues undergoing elongation and differentiation, but not lignification. At the 14th leaf stage, maize internodes that were showing high PAL activity (I13 - I14) were younger tissue, still elongating and differentiating, prior to lignification. Older tissue of the lower internodes (I7 - I9) had finished elongating and

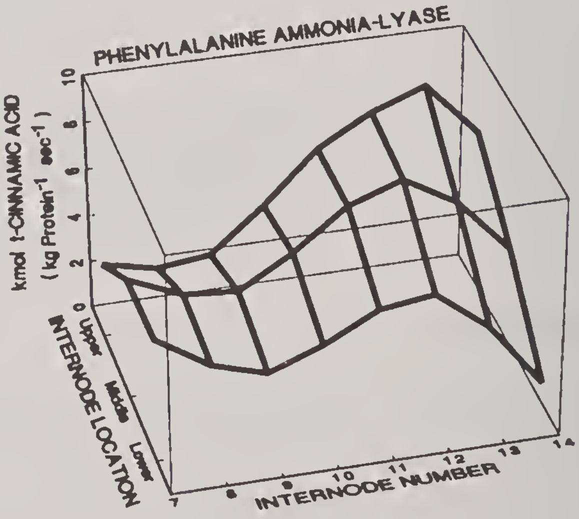


Figure 1. PAL activity in lower, middle, and upper maize internodes 7 through 14.

were lignifying (Fig. 1). PAL activity in these tissues had receded from earlier elevated activity. Correlations of PAL activity with CAD, CELL, HEMI, and ADL content were negative. CAD activity, on the other hand, was associated with tissue undergoing secondary cell-wall development after cessation of elongation. Internodes showing high CAD activity (I7 - I11) were lignifying, following elongation. Internodes which were still elongating (I12 - I14) expressed low CAD activity (Fig. 2). ADL content closely followed CAD activity (Fig. 2). CAD activity was positively associated with CELL (r = .76), HEMI (r=0.58), and ADL (r = .68) contents.

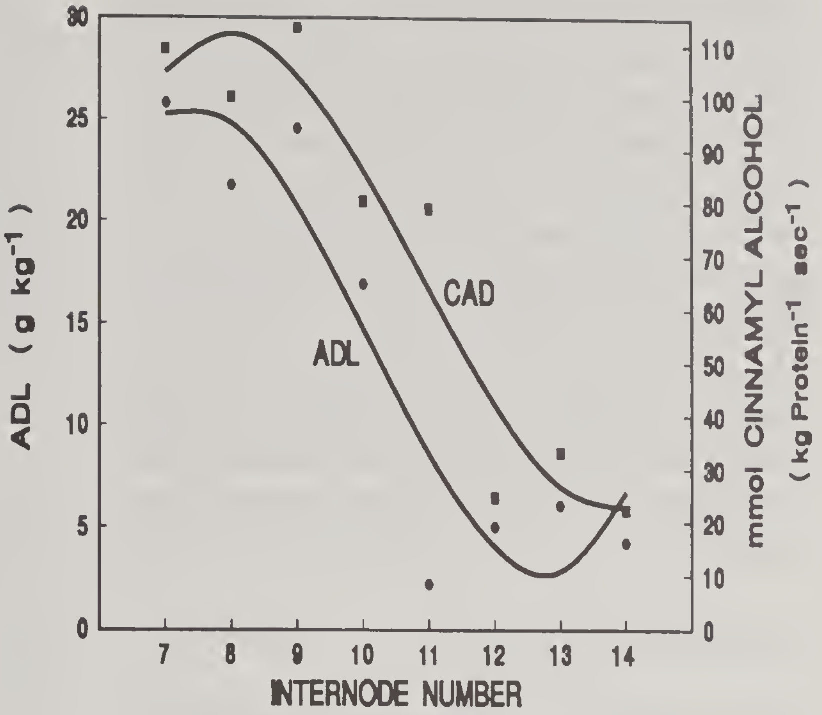


Figure 2. ADL content and CAD activity in maize internodes 7 through 14. Internode location was not significant.

Conclusion

Results suggest that CAD may have a regulatory role in the lignification process since its activity correlates well with cell-wall fiber content. PAL (and TAL) appears to supply the cinnamic acids for lignin biosynthesis without regulating their use. Forage breeders may target CAD as an enzyme to modify in efforts to reduce the negative effects of lignin on forage digestibility.

ACTIVITY OF MONOLIGNOL-SYNTHESIZING ENZYMES AND LIGNIN CONTENT IN A DEVELOPING MAIZE INTERNODE

T.A. MORRISON, J.R. KESSLER, R.D. HATFIELD and D.R. BUXTON

Introduction

Information about the process of lignification may shed light on how to manipulate the biosynthetic pathways in efforts to reduce to negative effects of lignin on forage digestibility. Lignification during secondary cell-wall development indirectly involves the enzymes of the general phenylpropanoid pathway, including phenylalanine ammonia-lyase (PAL) and directly involves enzymes, such as cinnamyl alcohol:NADPH dehydrogenase, of the lignin pathway which follows. PAL expression is regulated by events taking place during plant development and produces cinnamic acid precursors used in the lignin pathway, but it does not seem to be directly involved in lignin biosynthesis. CAD activity, on the other hand, may be specifically regulated by factors within lignin metabolism and it may directly influence synthesis of the lignin final product. CAD reduces cinnamyl aldehydes, produced via intermediate enzymes acting between PAL and CAD along the phenylpropanoid and lignin pathways, to cinnamyl alcohols that are condensed and polymerized by cell-wall peroxidases to lignin.

Previous studies indicate that PAL and CAD act in a coordinated, sequential fashion: PAL activity was high in growing tissue and declined in maturing tissue, superseded by CAD activity, which rose in maturing tissue and declined after lignification was well advanced. Cell-wall lignin content was low in material with low CAD activity and rose in material with high CAD activity. The objective of this study was to follow more closely the activities of PAL and CAD and the deposition of lignin during the temporal development of one maize internode.

Materials and Methods

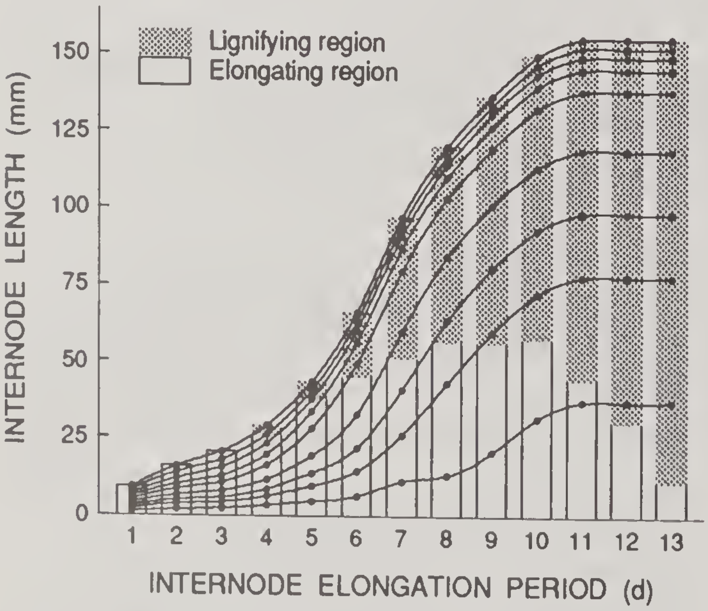


Figure 1. Regions of elongation and lignification in developing Internode 10. Spline plots indicate the incremental increase in space between paint dots originally placed 1-mm apart. Shaded bars indicate where lignin has been deposited.

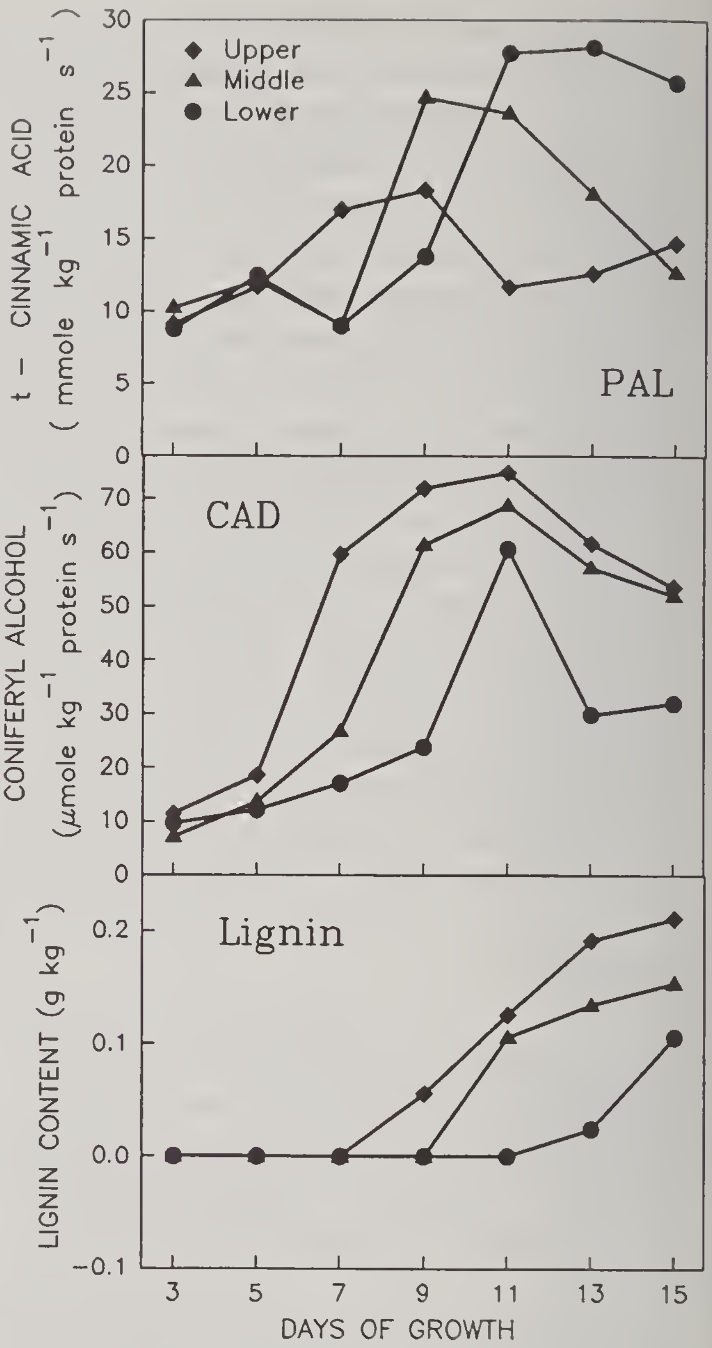


Figure 2. Activity of PAL, CAD, and Lignin content in lower, middle, and upper sections of maize Internode 10 harvested 3, 5, 7, 9, 11, 13, and 15 d after the internode had reached 10-mm in length.

Maize seedlings of single-cross hybrid Mo17 ´ B73 were grown in a growth chamber. When Internode 10 (I10) reached 10-mm in length, a 5-mm x 20-mm window was cut through the sheath of Leaf 9 to expose it. I10 was dotted along its length with black acrylic paint at 1-mm intervals. I10 was photographed every 24 h for 13 d to document in 'life-size' photographs daily incremental internode elongation. The basipetal progression of lignification in developing I10 was documented by daily photographing phloroglucinol-HCl stained cross-sections of the internode from similar-stage plants. I10 from greenhouse-grown maize plants were harvested 3, 5, 7, 9, 11, 13, and 15 d after I10 had reached 10-mm in length. I10 samples were excised, divided into lower, middle and upper section composites, frozen at -20°C, and freeze-dried. I10 section composites were ground, 250 mg samples extracted in Tris-HCl/2-mercaptoethanol/PEG buffer. Samples were centrifuged and supernatants assayed for PAL and CAD activity. PAL activity was assayed by the Abell and Shen method, CAD was assayed by the Wyrambik and Grisebach method. Pellets were washed and filtered in sequence with extraction buffer, water, 80% ethanol, and 2:1 chloroform/methanol solution. Samples received acid detergent fiber and acid detergent lignin treatments by the Goering and Van Soest method and acetyl bromide lignin treatment by the Iiyama and Wallis method. Lignin content was calculated by their equation.

Results and Discussion

PAL and CAD activity patterns and lignin content patterns described a coordinated, sequential, and basipetal wave pattern through the upper, middle, and lower internode sections, spanning the stages of cellular development and secondary cell-wall formation. The PAL activity increased during internode development as enzyme expression passed basipetally from upper to middle to lower internode regions over time; highest activity was maintained in internode sections undergoing elongation and differentiation with peak activity occurring in the lower internode section on day 13. The CAD activity rose to a peak on day 11 in the upper internode region and maintained greatest activity there even while CAD expression extended into middle and lower internode regions, as maturation events superseded elongation activity there. Lignin accumulated first in upper, then middle, and lastly in lower internode regions following high levels of CAD in those regions. Approximately two days elapsed between measurement of high CAD activity in an internode region and appearance of lignin in that region. Lignin content correlated with CAD activity (r = 0.53) but not with PAL activity (r = 0.16).

Conclusion

PAL is most expressive in the elongating and differentiating tissues while CAD is most expressive in maturing tissue. Lignin is deposited in a pattern following that of CAD activity indicating that CAD influences synthesis of the final lignin product. This information should be of value to forage breeders who may target CAD as an enzyme to manipuate in efforts to reduce to negative effects of lignin on forage digestibility.

FACTORS AFFECTING DRY MATTER DETERMINATION IN FEEDS

D.R. MERTENS

Introduction

Dry matter (DM) concentration is the most critical chemical analysis measured on feeds because: (1) it is used to adjust all other analyses to a DM basis, (2) each 1% DM represents 20 lbs of water per ton that is purchased at the price of the feed and (3) it affects the weight of as-fed quantities mixed in rations, thereby altering the nutrients actually given to the animal. Although it seems that DM should be simple and easy to measure, it is usually the determination that is most variable among laboratories. This is due primarily to the number of drying methods used and the

David R. Mertens

lack of accepted official standard methods for measuring DM. In addition, drying is necessary to prepare samples for grinding and analysis, and methods used to dry samples for analysis or DM determination are not compatible. To remove all the water from a sample, it must be dried at temperatures exceeding 100°C for several hours. However, drying at these temperatures creates artifact lignin and heat-damaged protein that interfere with fiber analyses.

The only AOAC method for measuring DM in air-dry feeds is oven drying at 135°C for 2 hr. This method is inconvenient for many laboratories because the time of drying must be exact, this oven temperature is not compatible with those used for fiber analyses and it cannot be used to prepare samples because it results in artifact lignin. The objectives of this research were to evaluate a two-step drying procedure for preparing samples and determining DM and to evaluate drying methods at 100 and 105°C that would be compatible with oven temperatures used for fiber analyses.

Methods

Four wet samples (two alfalfa samples, corn silage and high moisture corn) were used to evaluate DM methods. All samples were partially dried at 55°C for 16 hrs. One set (Set 1) of four samples was dried for an additional 32 hrs (48 hrs total) at 55°C then dried for 24 hrs at 105°C. Set 1 was used to determine total DM of the samples directly. After partial drying, Set 2 was weighed to determine partial DM, immediately ground and a laboratory DM (24 hrs at 105°C) was determined. After partial drying, Set 3 was exposed to a typical laboratory room environment (23.6°C and 49% relative humidity) before it was ground and a laboratory DM was determined. Set 4 was exposed to a hot, humid laboratory environment (29°C and 74% relative humidity) before it was ground and analyzed for laboratory DM. Sets 3 and 4 were also used to evaluate laboratory DM determinations at 100, 105 and 135°C.

Results and Discussion

It is recognized that oven drying of fermented samples results in the loss of volatile acids, alcohol and some ammonia. For this reason Karl Fischer or toluene distillation (corrected for non-water volatiles) are the most accurate DM methods for these type of feeds. However, dried samples of fermented feeds must be used for other analyses and these results are corrected to oven-dried laboratory DM basis. This research was designed to evaluate the errors in this latter DM approach.

Errors can occur in the two-step drying method if the sample is allowed to gain moisture between the times that partial and laboratory DM's are determined. Partial DM changed from approximately 47% when weighed immediately after removal from the oven to 48.8 or 49.6% when exposed to typical or humid environments, respectively (Table 1). In this experiment, the gain in moisture was determined, but most laboratories routinely weigh the samples immediately after removal from the oven. Thus the moisture gained by the partially dried sample would be unknown, but would be included in their laboratory DM analysis. Total DM is determined by multiplying the partial DM times the lab DM. For Set 2, this result agrees with the directly measured DM in Set 1. However, if partial DM is measured immediately, but the samples gain moisture before the lab DM is measured, total DM (42.9% for Set 3 or 42.7% for Set 4) will underestimate the directly measured DM in Set 1. This error can be avoided if partial DM is measured after 2 to 4 hrs exposure to the laboratory environment (Table 1).

Partially dry samples were oven dried in porcelain crucibles or aluminum pans for 16 or 24 hrs at 100° or 105°C to determine the effects of drying vessel, time and temperature on laboratory DM. Samples dried more thoroughly in aluminum pans because the surface area of samples was greater in those vessels than in crucibles (Table 2). Lengthening drying time from 16 to 24 also resulted in lower DM, as did drying at the higher temperatures (Table 2). Drying samples for 16 hr at 105°C resulted in the same DM as drying for 24 hrs at 100°C. Similarly, drying at 135°C is more rapid than at 105°C (Table 3). For NDF residues, it appears that drying for 2 hr at 135°C is equivalent to 4 to 6 hrs at 105°C. It also appears that DM determined at 105°C for 24 hr is only slightly lower than the DM determined at 135° for 2 hr (Table 3).

Conclusion

It is recommended that laboratory DM can be routinely determined on air-dry samples by drying for 24 hrs at 105°C or by drying at 135°C for 2 hrs. Other research suggests these methods will duplicate DM determined by the Karl Fischer methods for air-dry samples. Dry matter for wet, fermented feeds should be determined by Karl Fischer or toluene distillation (corrected for volatiles). Where the two-step approach is used to routinely prepare samples and determine DM, it is important to expose the sample to the laboratory environment for 2 to 4 hrs before the partial DM is measured to eliminate errors.

Table 1. Differences in total dry matter concentration when partial dry matter determinations were weighed immediately or allowed to absorb moisture in typical or humid laboratory conditions.

Dry Matter Type/Conditions	Set 1 Direct	Set 2 Immediate	Set 3 Typical	Set 4 Humid
	- - - - - - - -	- - - - - (% DM)	- - - - - - - -	- - - - - - - -
Partial DM/Immediate	(47.3)	47.3	47.0	47.2
Partial DM/Exposed			(48.8)	(49.6)
Lab DM		94.0	91.3	90.4
Total DM/Immediate	44.5	44.5	42.9[a]	42.7[a]
Total DM/Exposed			(44.6)[b]	(44.8)[b]

[a] Total DM calculated using partial DM from an immediately weighed sample.

[b] Total DM calculated using partial DM after 4-6 hrs exposure to laboratory environments.

Table 2. Effect of oven temperature, drying and weighing vessel on dry matter determination.

Oven temperature	100°C		105°C	
Drying time:	16 h	24 h	16 h	24 h
Vessel/Sample type	% DM	% DM	% DM	% DM
Porcelain crucibles				
Immediate	94.5	94.2	94.2	93.9
Typical environment	92.0	91.6	91.6	91.2
Humid environment	90.9	90.6	90.6	90.3
Aluminum pans				
Humid environment	90.4	90.1	90.1	89.7
Average	91.9	91.6	91.6	91.3

Table 3. Changes in dry matter determination with increasing drying time at two oven temperatures for intact air-dry samples DM and neutral detergent fiber (NDF) residues.

Drying time	DM		NDF	
(hr)	105°C	135°C	105°C	135°C
	------- (% of initial sample weight) --------			
1		90.7		41.8
2	92.5	90.2		41.8
3	92.3	89.8	41.9	41.7
4	92.2	89.6	41.8	41.5
6	91.9		41.6	
8	91.7		41.6	41.4
24	90.8		41.5	

BUFFERING SYSTEM CAPABLE OF MAINTAINING CONSTANT pH BETWEEN 5.8 AND 6.8 DURING IN VITRO FERMENTATION

R.J. GRANT and D.R. MERTENS

Introduction

Fiber digestion is usually reduced when moderate to high levels of readily fermentable carbohydrates are included in rations for cattle and sheep. However, little is known about the mechanism causing reduced fiber digestion or the specific aspects of fiber digestion kinetics that are affected. This reduction could be caused by a direct effect of fermentable carbohydrate on fiber digestion or by an indirect effect associated with low pH when carbohydrates are rapidly fermented. Research suggests that starch addition to forages in an in vitro system in which pH was maintained at 6.8 did not result in changes in fiber digestion kinetics that could explain the large reductions in fiber digestion that are observed in animals. This suggests that ruminal pH may play a key role in depressing in forage fiber utilization. The potential for negative effects of pH on fiber digestion exists in high producing dairy cows that are typically fed rations containing 50-60% concentrates. The objective of this research was to develop a buffering system capable of maintaining constant pH's in the range between 5.8 and 6.8 during in vitro fermentation.

Methods

The in vitro fermentation system of Goering and Van Soest (USDA Handbook 379, 1970) that uses continuous gassing of each flask by CO_2 was modified by varying the times of fermentation and the buffer media. The original McIlvaine solution was modified to contain bicarbonate. The pH 5.8 McIlvaine - bicarbonate media contained 43.9 ml of 0.1M citric acid, 49.5 ml of 0.2M Na_2HPO_4 and 6.6 ml of bicarbonate solution described by Goering and Van Soest, (GVS), whereas the pH 6.2 buffer contained 21.5 ml of 0.1M citric acid, 72.8 ml of 0.2M Na_2HPO_4 and 5.7 ml of GVS bicarbonate solution. The phosphate-bicarbonate-citric acid buffer solutions contained 960 ml of total buffer solutions described by GVS and 40 ml of 1.0M citric acid for pH 5.8 or 982 ml GVS total buffer solution and 18 ml of 1.0M citric acid for pH 6.2. The original total buffer solution of GVS was used for pH 6.8.

Results and Discussion

Several buffering systems were evaluated. The Good et al. (1966) buffers typically used to buffer biological systems (MES, PIPES) were evaluated but did not maintain pH adequately during fermentation. McIlvaine solution which contains citric acid and dibasic phosphate can be prepared to maintain pH in the range desired. Initial experiments indicated that bicarbonate is a crucial component in any in vitro ruminal buffering system kept anaerobic under continuous CO_2 pressure. However, McIlvaine solution with bicarbonate did not maintain desired pH beyond 24 h of fermentation (Figure 1). Because we were not able to maintain pH, especially at pH 6.8, no NDF digestion experiments were performed using McIlvaine's buffers.

A more direct approach to developing a buffer system was developed by adjusting the in vitro buffer system (GVS) of Goering and Van Soest (USDA Handbook 379, 1970) using citric or phosphoric acids. Addition of both acids to the GVS buffer were evaluated at pH

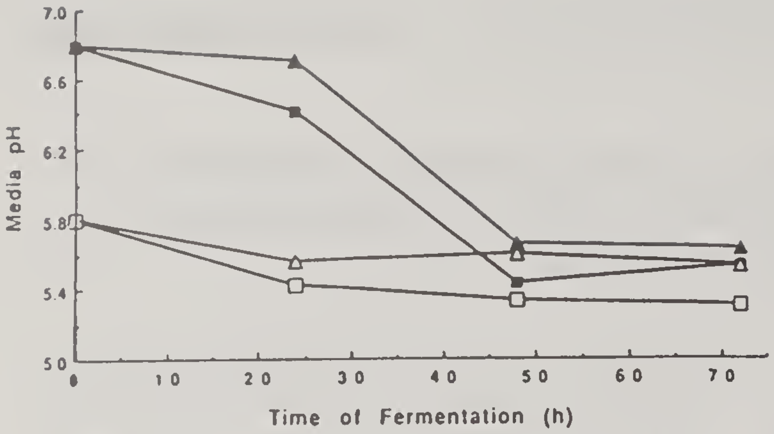


Figure 1. Change in pH of fermentation media after various times of fermentation when McIlvaine's solution and bicarbonate mixture were used as the in vitro buffer system. Substrates included timothy at pH 5.8 (□), timothy at pH 6.8 (■), alfalfa at pH 5.8 (Δ), and alfalfa at pH 6.8 (▲). Note ineffectiveness of buffer system for maintaining pH after 24 h of fermentation.

5.8, 6.2 and 6.8. To determine if acid solutions altered fermentation by effects other than pH change, GVS buffer was reduced in pH to 5.2 with each acid and readjusted to pH 6.8 with bicarbonate. Neither citric nor phosphoric acid affected fiber digestion at pH 6.8 (Table 1). Both citric and phosphoric acids obtained similar NDF residue weights for all fermentation times observed at pH 5.2, 5.8 and 6.8. The pH of flasks was measured at 0, 4, 12, 24, 48 and 72 h and adjusted with bicarbonate if necessary. Citric acid maintained pH at desired levels with less bicarbonate addition (0 to 3.0 mL) than phosphoric acid (1.5 to 5.0 mL). This agrees with other research that reported difficulty in maintaining constant pH using phosphoric acid.

Conclusion

Given that citric acid exerted no apparent negative effect on NDF digestion except via pH change and that it maintained constant pH more completely than phosphoric acid, we will use the phosphate-bicarbonate-citric acid buffer system in future experiments to study the effects of pH on fiber digestion kinetics.

Table 1. In vitro residues as affected by citric (C) and phosphoric (P) acid additions to Goering and Van Soest (GVS) in vitro buffers when readjusted to pH 6.8 with bicarbonate.

	Alfalfa silage			50% Alfalfa silage + 50% Corn		
Time	GVS	GVS + C	GVS + P	GVS	GVS + C	GVS + P
	- - - - - - - - - - - - - - - -(NDF residue on initial DM basis) - - - - - - - - - - - - - - - - - - -					
(h)						
0	38.5	39.2	39.3	28.0	28.6	27.1
12	31.0	30.5	29.1	18.0	18.4	18.9
24	26.0	24.4	24.6	14.5	14.2	13.5
48	20.2	20.8	20.6	11.1	12.0	11.4
72	19.3	19.5	20.4	9.5	10.9	10.8

ALTERED FIBER DIGESTION KINETICS ASSOCIATED WITH LOW pH AND RAW CORN STARCH ADDITION

R.J. GRANT and D.R. MERTENS

Introduction

As the proportion of concentrate in rations is increased to meet the energy requirements of high producing dairy cows, fiber digestion is reduced which may be associated with lower ruminal pH or starch feeding. Dairy cows fed 24 kg/d of concentrate containing 32% starch had an average ruminal pH of 6.1. Optimal pH for ruminal microorganisms ranges between 6.5 to 6.8. Thus, fiber digestion may be depressed by the negative effects of pH on fiber-digesting ruminal microorganisms. It may also be possible that fiber digestion is impaired because more readily fermentable carbohydrates are degraded preferentially. Research suggests that bacterial affinities for carbohydrates vary and that easily fermented carbohydrates such as sugars and starches may be used before fibrous carbohydrates are degraded. The objective of this experiment was to determine the independent and combined effects of starch addition and ruminal pH on fiber digestion kinetics using a phosphate-bicarbonate-citric acid buffer system developed in our laboratory.

Methods

The in vitro procedure used was a modification of that of Goering and Van Soest (USDA Handbook 379, 1970). Substrates used were alfalfa hay (AH) containing 45% NDF (ash-free, DM basis), brome hay (BH) containing 67% NDF, corn silage (CS) containing 39% NDF, 66% AH + 34% raw starch (AHC) and 44% BH + 56% raw corn starch (BHC). The latter two substrates were formulated to contain 30% NDF in the mixture.

The model used to describe kinetics of fiber digestion was:

$$Y = D_o e^{-k_d(t-L)} + INDF;$$

where, Y = NDF residue at time t, D_o = potentially digestible NDF (% of initial DM), k_d = fractional rate constant of digestion (h^{-1}), L = discrete lag time (h), t = time (h), and INDF = indigestible NDF (% of initial DM).

The model was initially fitted to the data using logarithmic transformation and linear regression. The parameters from this procedure were used as initial estimates for deriving final parameter estimates using nonlinear regression. Potential extent of NDF digestion (PED) was calculated as:

$$PED = 100 \times [D_o / (D_o + INDF)].$$

Ruminal digestibility of NDF was calculated using the equation:

$$RD = PED \times e^{-k_p L} \times [k_d/(k_d + k_p)];$$

where, RD = ruminal digestibility of NDF (%), k_p = rate of passage of fiber particles, using a constant of .02 h^{-1}, and all other terms as defined previously.

Results and Discussion

When averaged across all substrates, pH 5.8 resulted in longer lag times (7.47 h) and slower fractional rates of digestion (.049 h^{-1}) than pH 6.2 (4.01 h and .073 h^{-1}, respectively) or pH 6.8 (3.31 h and .074 h^{-1}, respectively). Treatments that did not contain starch (AH and BH) had faster fractional rates of digestion and longer lag times (Table 1) than those that did (AHC, BHC and CS). The effects of starch addition on fiber digestion kinetics were more consistent when expressed as a proportional response. Fractional rates, lag times and PED of starch-containing substrates were 84, 78 and 77% of substrates containing no starch. There were significant substrate by pH and substrate by starch addition interactions. For AH, lowering pH reduced fractional rate, but did not change lag; whereas for BH lowering pH increased lag time but did not change rate.

When substrates contained starch (AHC, BHC and CS), reducing pH consistently decreased fractional rates of digestion and increased lag times.

The combined effects of kinetic changes from added starch and lowered pH is difficult to ascertain without resorting to a model that integrates lags and rates of digestion with rates of passage. Predicted ruminal digestibility of NDF is given in Table 2. When expressed as a percentage of the system buffered at pH 6.8 with or without added starch, it appears that alfalfa and grasses react differently. For BH, it appears that effects of pH and starch are consistent and multiplicative. The effect of lowering pH on RD is the same with or without starch and is similar to the effect of pH on CS. Although AH shows a similar response to BH, BHC and CS when starch is added, this substrate is affected by pH only slightly when no starch is added. The combined effects of starch and pH appears to be similar for both AH and BH.

Conclusions

The buffer system that maintains constant pH at levels other than 6.8 allows the separation of direct starch and indirect pH effects on fiber digestion kinetics. When substrates contained corn starch, lowering pH consistently resulted in a decrease in fractional rate of digestion and an increase in discrete lag times. When no starch was added, lower pH resulted in decreased rate and no change in lag for alfalfa hay, but resulted in increased lag and no change in rate for brome hay. Research is needed to confirm if this is a consistent attribute of grasses and legumes. When changes in digestion kinetics are translated into ruminal digestibilities, the results of these in vitro studies compare well with observed responses in fiber digestion by animals.

Table 1. Effect of forage, starch, and pH on kinetics of NDF digestion.[1]

				Digestion parameters		
Forage	Starch[2]	pH	Lag	Rate	INDF[3]	PED[4]
			(h)	(h^{-1})	------ %	-------
Alfalfa hay	-	6.8	4.12[b]	.106[a]	25.5[a]	43.0
	-	6.2	4.83[b]	.112[a]	25.4[a]	43.6
	-	5.8	4.81[b]	.062[c]	24.4[a]	45.9
	+	6.8	1.80[c]	.090[ab]	20.3[b]	33.1
	+	6.2	2.65[c]	.080[bc]	19.7[b]	34.0
	+	5.8	7.06[a]	.066[c]	19.9[b]	33.8
Bromegrass hay	-	6.8	4.58[c]	.066[a]	28.5[a]	57.3
	-	6.2	5.05[bc]	.057[a]	29.3[a]	56.5
	-	5.8	9.90[a]	.050[a]	31.1[a]	53.6
	+	6.8	3.59[c]	.062[a]	17.1[b]	43.1
	+	6.2	3.59[c]	.055[a]	16.3[b]	44.0
	+	5.8	7.01[b]	.032[b]	16.6[b]	44.7
Corn silage	+	6.8	2.47[c]	.045[bc]	11.1[b]	71.4
	+	6.2	4.68[b]	.060[a]	13.8[a]	64.0
	+	5.8	8.56[a]	.033[c]	12.7[ab]	67.0

[a,b,c] Means within a column and forage combination differ ($P < .05$).
[1] Each value is the mean of three observations.
[2] With (+) or without (-) starch in the substrate.
[3] Indigestible NDF (percentage of initial DM).
[4] Potential extent of NDF digestion (percentage of total NDF).

Table 2. Illustration of potential impact of starch and pH differences on predicted ruminal NDF digestibilities.[1]

Forage	Starch	pH	RD[2]	RD Ratio pH[3]	RD Ratio pH + starch[4]
			(%)	— (% of RD at pH 6.8)—	
Alfalfa hay	-	6.8	33.1	100.0	
	-	6.2	33.6	100.8	
	-	5.8	33.5	94.6	
	+	6.8	26.1	100.0	78.4
	+	6.2	25.8	98.7	77.4
	+	5.8	22.5	86.2	67.6
Bromegrass hay	-	6.8	40.1	100.0	
	-	6.2	37.8	94.2	
	-	5.8	31.4	78.3	
	+	6.8	30.3	100.0	75.6
	+	6.2	30.0	99.0	74.8
	+	5.8	23.9	78.8	59.6
Corn silage	+	6.8	47.0	100.0	
	+	6.2	43.7	92.9	
	+	5.8	35.2	74.7	

[1] Calculated using data from Table 1 and a rate of passage of .02 h^{-1} for fiber particles.

[2] Predicted ruminal NDF digestibility = PED x e^{-k_pL} x $k_d/(k_d + k_p)$.

[3] Effect of pH on ruminal digestibility (RD) ratio relative to RD at pH 6.8

[4] Effect of pH and starch on RD ratio compared to RD at pH 6.8 without starch.

EFFECTS OF pH AND GROWTH RATE ON THE FERMENTATION OF CELLULOSE BY THE RUMINAL CELLULOLYTIC BACTERIUM *Fibrobacter succinogenes* S85

P.J. WEIMER

Introduction

Fibrobacter succinogenes is generally regarded as the most abundant cellulolytic bacterium in the rumen of animals fed poor-quality forage. The nutritional characteristics and intermediate catabolic pathway of this species have been well-characterized, as have a number of its more abundant cellulolytic enzymes. However, there is almost no quantitative data on cellulose degradation or fundamental growth parameters (e.g., cell yields or maintenance coefficients) of this organism, particularly under controlled growth condi-

Paul J. Weimer

tions. A study was thus undertaken to measure cellulose degradation, product formation, and cell yields as a function of pH and microbial growth rate.

Methods

F. succinogenes S85 was grown in a pH-controlled continuous culture reactor using a modified Dehority's medium with microcrystalline cellulose (4.8 g Sigmacell 20 per liter) as the growth-limiting nutrient. The medium containing the insoluble cellulose was continuously dispensed as a slurry through a peristaltic pump by CO_2-segmentation to prevent settling of cellulose in the delivery lines. Constant reactor volume of 940 ml was attained by gravity overflow of excess media. System steady state was normally attained after 3-5 dilutions, at which point samples were collected for measurement of cellulose (modified neutral detergent method), soluble and reducing sugars (anthrone and dinitrosalicylic acid methods), cell mass (from total N determined by Dumas combustion of washed cell pellets), and fermentation products (by HPLC). Twenty-four separate runs were made within the dilution rate (D) range of 0.015 - 0.076 h^{-1} and pH range of 6.11-6.85.

Calculated data for fractional cellulose consumption (cellulose consumed/cellulose fed), soluble sugars, molar yields of individual fermentation products, and cell yield were each fit to a response surface equation, using the General Limiting Model of the SAS statistical software package. The response surface equation used was: parameter = $\alpha + \beta D + \gamma P + \delta D^2 + \varepsilon P^2 + \phi DP + \eta D^2P + \varphi DP^2 + \kappa$, where α = constant, $\beta....\varphi$ = coefficients, D = dilution rate, P = pH, and κ = random error term.

Results and Discussion

Microscopic observations revealed that cellulose particles were completely colonized by microbial cells during steady state growth. Even though 19-42% of the cellulose was not consumed in the chemostats, the cultures were in fact limited by the amount of available cellulose surface. Despite this strain's reputation as the most active degrader of crystalline cellulose, the maximum dilution rate obtained on this substrate was 0.076 h^{-1}, well below the maximum D obtained in previous studies with *R. flavefaciens* FD-1 in the same culture media.

The response surfaces for various fermentation parameters are shown in Fig. 1. Fractional cellulose consumption was optimal at low D and varied little with pH in the growth range of the organism, although steady states could not be attained at pH values outside the range of 6.1-6.9. Replotting of predicted cellulose consumption versus retention times (1/D) at constant pH yielded nonlinear plots, suggesting that cellulose consumption is not first-order with respect to cellulose concentration at low growth rates. This may be due to a repression of cellulase synthesis in response to the relatively high maintenance requirements associated with slow growth, or may simply reflect the reduction in cellulose particle size (and thus total surface area) at longer retention times.

Cellulose digestion was accompanied by fairly high concentrations (up to 1 mM) of residual soluble sugar. The soluble sugars were primarily free glucose and were present at concentrations about three times higher than those observed in previous studies with *R. flavefaciens* FD-1. This observation is surprising in view of the fact that S85 will grow on glucose in batch culture while FD-1 will not; this in turn suggests differences in the hydrolytic specificities of each strain's cellulolytic apparatus and/or their affinities for transport of hydrolytic products. Yields of succinate and acetate showed only slight shifts with changing D or pH, suggesting minimal regulation of individual enzymes of intermediary catabolism.

Cell yields for strain S85 were normally in the range of 0.14 to 0.22 g cells/g cellulose consumed and generally increased with increasing growth rate (equivalent to dilution rate at steady state) due to the smaller fraction of energy consumed for maintenance requirements. Pirt plots of 1/ [predicted cell yield] versus the inverse of dilution rate were calculated from the response surface equation at each of several pH values. These plots revealed that maintenance coefficients were 0.04 - 0.05 g cellulose consumed / g cells / h, and changed only slightly with pH. These maintenance coefficients are significantly lower than those of other ruminal cellulolytic bacteria.

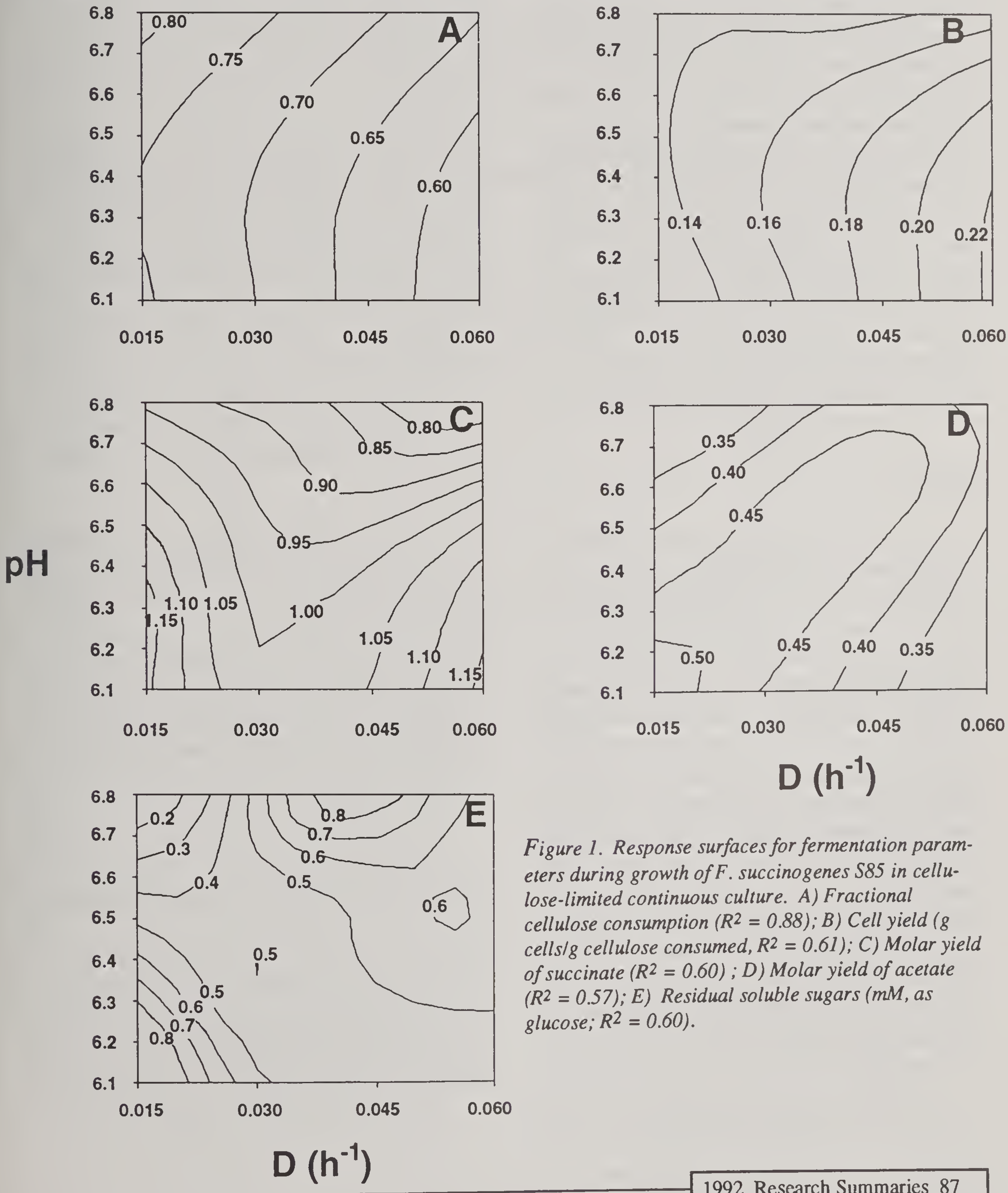


Figure 1. Response surfaces for fermentation parameters during growth of F. succinogenes S85 in cellulose-limited continuous culture. A) Fractional cellulose consumption ($R^2 = 0.88$); B) Cell yield (g cells/g cellulose consumed, $R^2 = 0.61$); C) Molar yield of succinate ($R^2 = 0.60$) ; D) Molar yield of acetate ($R^2 = 0.57$); E) Residual soluble sugars (mM, as glucose; $R^2 = 0.60$).

REGULATION OF END PRODUCT FORMATION BY THE CELLULOLYTIC RUMINAL BACTERIUM *Ruminococcus flavefaciens* FD-1

Y. SHI, P.J. WEIMER and J. RALPH

Introduction

Ruminococcus flavefaciens is one of the major cellulolytic bacteria in the rumen. The major fermentation end products of this species have been reported to be acetate, succinate, and formate, with lesser amounts of H_2 and lactate. While some of the intermediary catabolic enzymes of this species have been identified, the complete pathway of end product formation has not been determined, and little is known of how growth conditions affect the relative distribution of these end products. A study was undertaken to fully characterize the catabolic pathway and determine how end product formation and levels of catabolic enzymes were affected by microbial growth rate and extracellular pH.

Methods

R. flavefaciens FD-1 was grown in cellulose- or cellobiose-limited continuous cultures in a modified Dehority's medium at 26 different combinations of pH and dilution rate (D, equivalent to growth rate at steady state). Fermentation end products were determined by HPLC, except for H_2, which was estimated from redox balancing. Partitioning of substrate into various end products was determined by response surface analysis. Cells were harvested under N_2 and stored at -50 C prior to preparation of cell extracts using a French pressure cell. Extracts were assayed for various enzymes, using methods described elsewhere. Metabolism of ^{13}C-labeled substrates was determined by growing cells in the presence of the desired labeled substrate and analyzing NaOH-treated culture supernatants by ^{13}C-NMR.

Results and Discussion

As in several other ruminal cellulolytic bacteria, phosphoenolpyruvate (PEP) was the branch point for synthesis of succinate and acetate (Fig. 1). Succinate was produced via a GTP-producing, CO_2-consuming PEP carboxykinase, along with malate dehydrogenase, fumarate hydratase, and fumarate reductase. Rapidly-growing cells also contained low but significant quantities of the ATP-consuming carboxylation enzyme pyruvate carboxylase. Acetate production from PEP proceeded via pyruvate kinase, pyruvate dehydrogenase, phosphotransacetylase, and acetate kinase. H_2 was produced from protons via a hydrogenase. ^{13}C-NMR and ^{14}C-pyruvate exchange data clearly indicated that formate was formed by CO_2 reduction rather than by pyruvate cleavage.

When assayed at high (and presumably saturating) concentrations of substrate, most enzymes in the acetate and succinate synthetic pathway were present at levels well in excess of those required for synthesis of product at the observed rate in the chemostats; one marked exception was PEP carboxykinase, whose levels barely accounted for succinate production at the observed rates in all eight of the extracts examined.

Fumarate reductase activities were not detected in cell extracts and could only be demonstrated in toluenized whole cells in the presence of the reduced methyl viologen, an artificial electron donor. Because such treatment dissipated the transmembrane proton gradient, it was not possible to measure coupling of fumarate reduction to ATP synthesis.

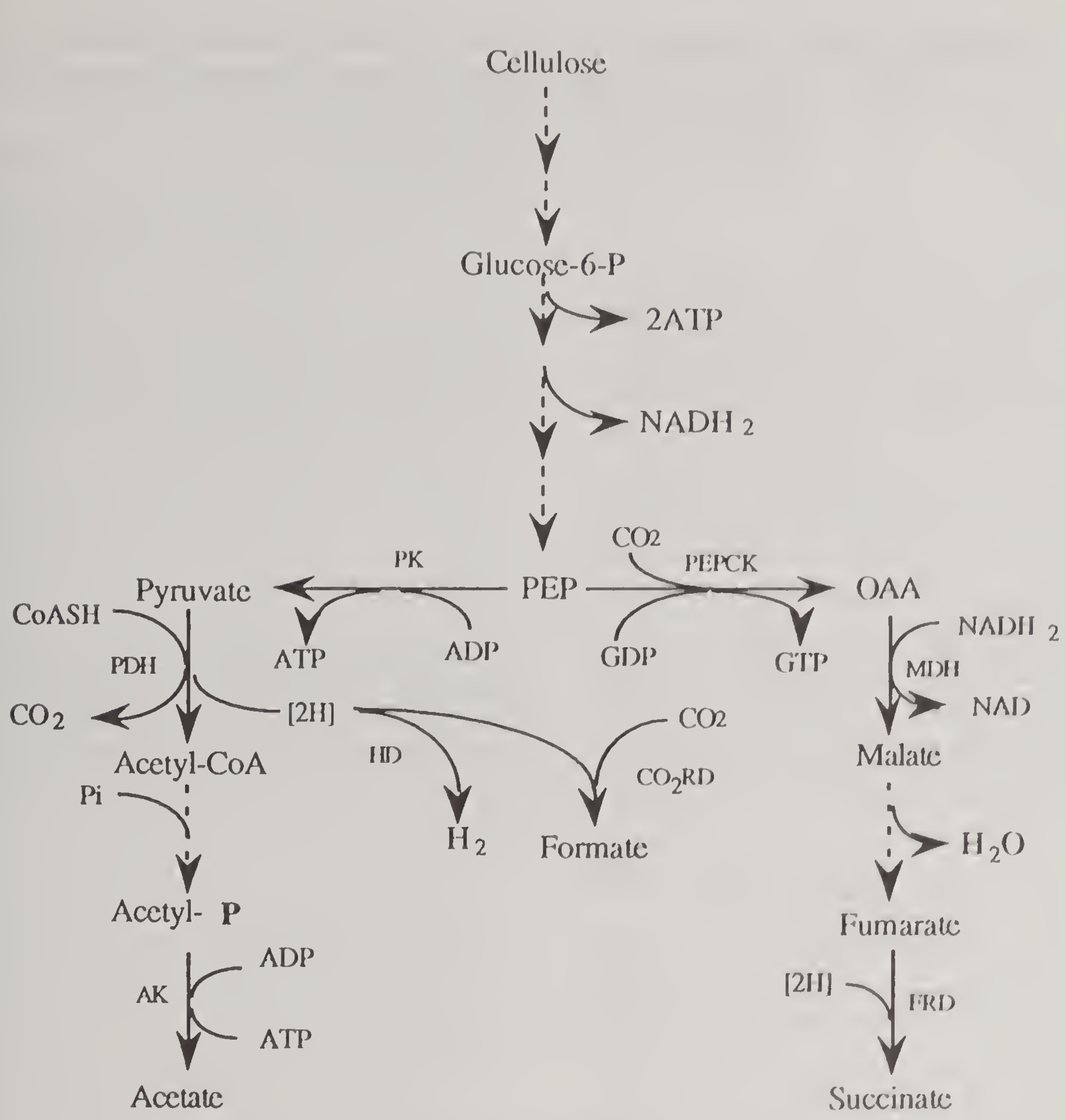

Cellulose
Glucose-6-P
2ATP
NADH 2
PEP
PK
Pyruvate
ATP
ADP
CO2
PEPCK
OAA
GDP
GTP
NADH 2
MDH
NAD
Malate
H2O
Fumarate
[2H]
FRD
Succinate
CoASH
PDH
CO2
[2H]
Acetyl-CoA
HD
H2
CO2
CO2RD
Formate
Pi
Acetyl- P
ADP
AK
ATP
Acetate

Figure 1. Intermediary catabolism in R. flavefaciens FD-1.

The overall energetic yield of the pathway thus remains uncertain. Production of acetate from PEP results in the conservation of two ATP (at pyruvate kinase and acetate kinase). Production of succinate from PEP results in conservation of one ATP at PEP carboxykinase; if fumarate reductase were coupled to ATP synthesis, the succinate pathway would have an ATP yield equivalent to that of the acetate path.

Consistent with the potential energetic equivalence of the two pathways was the observation that changes in pH or growth rate of *R. flavefaciens* FD-1 resulted in very little change in the relative flux of carbon to acetate and succinate. Moreover, approximately 60 per cent of the reducing equivalents generated during the fermentation were disposed of through succinate at all combinations of pH and D. However, major shifts were observed in the disposal of the remaining reducing equivalents (Fig.2), with formate production increasing at the expense of H_2 production as growth rate increased and pH deviated from the growth optimum of 6.6. Lactate was not produced by this strain, even at rapid growth rates, an observation consistent with the observed lack of lactate dehydrogenase in cell extracts at all growth rates.

The data suggest that succinate and acetate production by this organism are relatively fixed, and flux through the PEP branch point may be dictated by the concentration of PEP carboxykinase. Unlike many other ruminal bacteria (including some strains of *R. flavefaciens*), strain FD-1 regulates disposal of electrons through production of H_2 and formate, rather than through lactate production. This provides considerable benefit to the organism, since more carbon-containing fermentation intermediates would remain for participation in energy-yielding reactions of the acetate and succinate pathways.

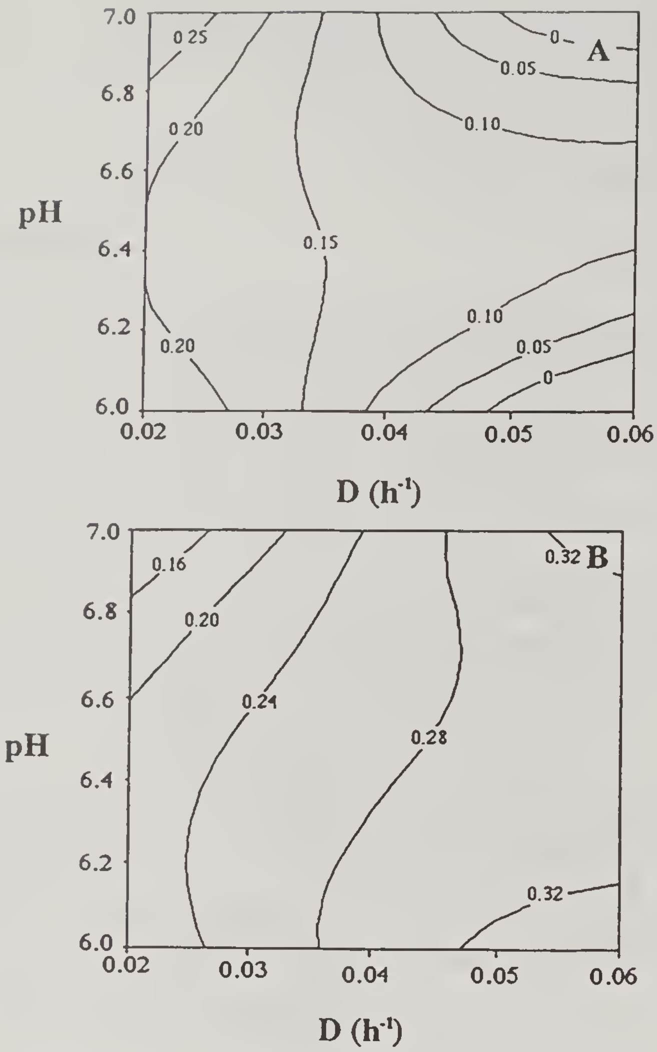


Figure 2. Response surfaces for partitionining of reducing equivalents into H_2 and formate by cellulose-limited continuous cultures of R. flavefaciens FD-1. A) Fraction of reducing equivalents disposed of as H_2 ($R^2=0.68$); B) Fraction disposed as formate ($R^2=0.63$).

Table 1. Product yields of *R. flavefaciens* FD-1 grown in cellulose- or cellobiose-limited continuous culture at pH ~ 6.8.

Substrate consumed			Molar yields of fermentation end products			
in chemostat (liter^{-1})	μ(h^{-1})	pH	Acetate	Succinate	Formate	H_2
3.11 g cellulose	0.020	6.80	0.820	0.590	0.297	0.590
3.25 g cellulose	0.035	6.75	0.766	0.568	0.580	0.044
3.28 g cellulose	0.059	6.83	0.846	0.685	0.684	-0.019
0.92 g cellulose	0.074	6.77	0.870	0.671	0.970	-0.150
1.05 g cellulose	0.244	6.75	1.106	0.506	1.431	-0.123

ORIENTED BINDING OF THE RUMINAL CELLULOSE BACTERIUM *Fibrobacter succinogenes* TO CRYSTALLINE CELLULOSE

P.J. WEIMER, C.L. ODT and Y. CHEN

Introduction

Fibrobacter succinogenes is one of the major cellulolytic bacteria of the rumen and has been reported to be the predominant cellulose degrader in ruminants fed poor-quality forage diets. As in the case of other major ruminal cellulolytic bacteria, active cellulose digestion by *F. succinogenes* requires intimate contact with the cellulose substrate. However, cellulose digestion of *F. succinogenes* is apparently unique in that it involves a distinct spatial orientation of cells on the fiber surface, and results in the formation of distinct parallel grooves on the surface of the partially-digested fiber. It has been proposed that this unique topological pattern is due to the orientation of cells along a discrete crystallographic plane, but the process has not been investigated in detail. We therefore examined the topology of the process using celluloses having different crystalline orientations along the fibrillar surface.

Materials and Methods

Two crystalline celluloses were used: ramie (cellulose from the bast fibers of *Boehmeria nivea)*, generously provided by A.D. French (USDA-ARS, New Orleans, LA), and ground cotton cellulose (Sigma CF-1, from cotton [*Gossypium hirsutum*]). Amorphous cellulose was prepared by dissolution of CF-1 in dimethylsulfoxide/sulfur dioxide/diethylamine, with subsequent regeneration in water, as described by Isogai and Atalla. The reprecipitated amorphous cellulose was exhaustively dialyzed against water to remove excess solvent prior to lyophilization. *F. succinogenes* S85 was grown in sealed serum vials under CO_2 using a modified Dehority medium amended with each type of cellulose. After 18-24 h incubation, cultures were treated with methylcellulose to remove most of the adherent cells, and the cellulose was recovered by filtration onto glass fiber filters. The filters containing the cellulose were extensively washed with water, passed through a graded series of ethanol/water, ethanol and acetone solvents, then was critical point dried under CO_2. After sputter coating with Au, samples were visualized with a JEOL JSM-35CF scanning electron microscope.

Results and Discussion

Digestion of crystalline celluloses of both ramie and cotton by *F. succinogenes* was accompanied by formation of discrete parallel grooves on the surfaces of the fibers. In the case of ramie, these grooves were always nearly parallel to the fiber axis, in accord with the known fact that the crystallographic axis of cellulose in ramie is always within 8° of the fiber axis. The grooves in cellulose displayed a different pattern: grooves were always parallel to one another on a particular fiber, but the groove angle with respect to the fiber axis was highly variable from fiber to fiber. This variation apparently reflects the known changes in orientation of cellulose during self-assembly of the cotton fibers as a helicoidal array of microfibrils. Cellulose digestion of amorphous cellulose resulted in the formation of randomly-oriented pits rather than distinct parallel grooves. This observation is consistent with the lack of a crystallographic plane in amorphous cellulose.

Taken together, the data verify the hypothesis that *F. succinogenes* adheres to and digests cellulose along a discrete crystallographic plane. This unique topological pattern may in part account for the purported superior ability of this microbial species to digest crystalline cellulose.

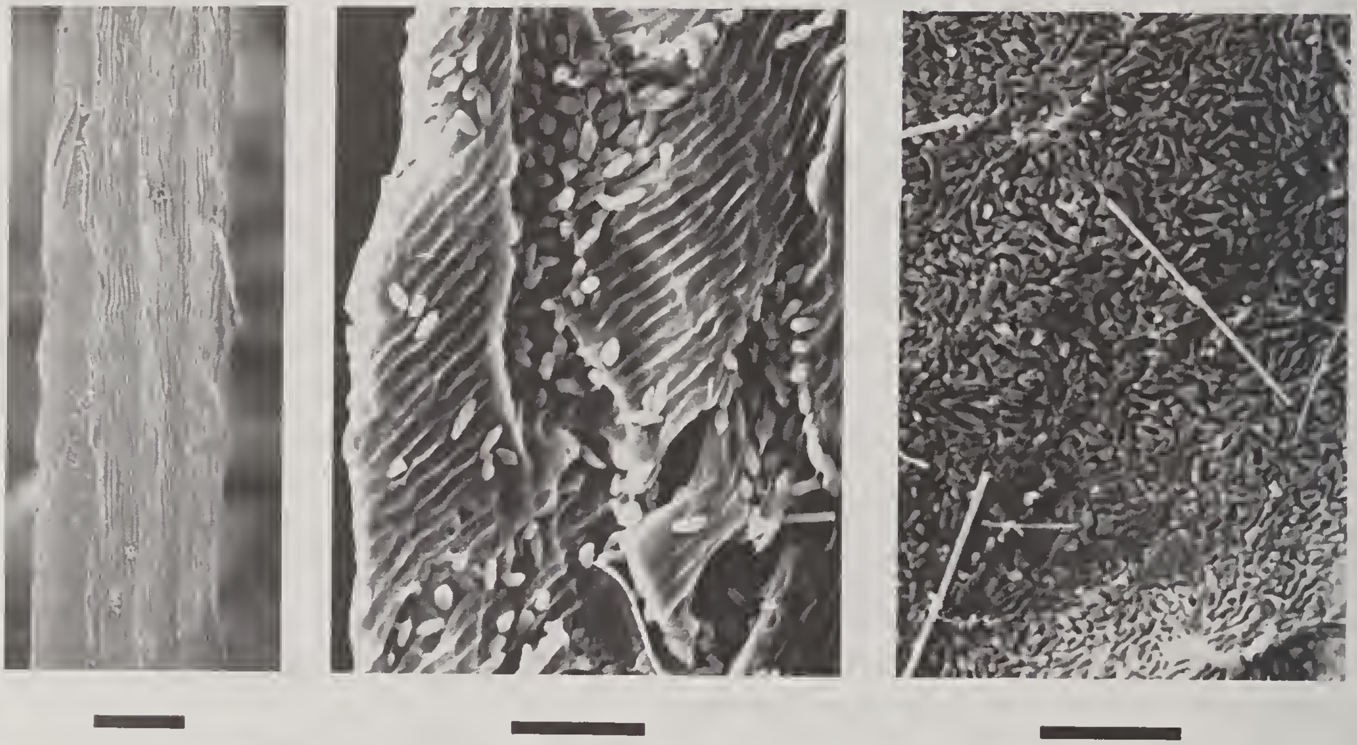


Figure 1. Electron photomicrographs of celluloses after partial digestion by F. succinogenes S85. Left: Ramie; Center: Crystalline (CF-1) cellulose; Right: Amorphous cellulose. In all panels, fiber axis is oriented vertical to the page. Bars represent 10 μm.

RUMINAL IN VITRO DEGRADATION OF PROTEIN FROM DIFFERENT FORAGE LEGUME SPECIES

K.A. ALBRECHT and G.A. BRODERICK

Introduction

Protein in many forage legumes is poorly utilized by ruminants because of extensive degradation during harvest and storage, followed by further degradation in the rumen. This degraded protein can be useful to the animal through the synthesis of protein by ruminal microbes. In high forage rations, however, low levels of digestible energy usually minimize the amount of NPN which can be recaptured in the form of microbial protein. Excess N is excreted through the urine and represents both a nutritional loss and a disposal problem. Excessive ruminal protein degradation is perhaps the most limiting nutritional factor in temperate forage legumes harvested at recommended maturity stages. Thus, there is interest in identifying factors that influence the amount of forage protein which survives harvest and storage and passes intact through the rumen into the lower digestive tract. Our objectives were to determine if variability existed among forage legumes for ruminal protein degradability and to identify what factors may be associated with this variability.

Materials and Methods

Forage legumes representing a wide range of species adapted to the temperate region were field harvested from triplicate plots then lyophilized in late summer in both 1989 and 1990. These samples later were ground and analyzed for tannins by a radial diffusion assay. Samples then were assayed for the rate of ruminal protein degradation and estimated escape using the limited substrate (LS) inhibitor in vitro procedure (Broderick, Brit. J. Nutr. 58:463, 1987). Most of the samples from 1990 also were assayed for degradation rate and estimated ruminal escape using a Michaelis-Menten (MM) approach with nonlinear regression analysis of the integrated Michaelis-Menten equation (Broderick and Clayton, Brit. J. Nutr. 67:27, 1992).

Results and Discussion

Tannin concentrations and in vitro protein degradabilities are in Table 1. In forage legumes harvested during 1989, fractional protein degradation rates and ruminal escapes (estimated with limited substrate methodology) ranged from .293/h and 16% for crownvetch to essentially 0 and 96% for sericea lespedeza [*Lespedeza cuneata* (Dum.) G. Don. cv. Serala 76]. Ruminal protein escapes estimated by the limited substrate method for samples harvested during 1990 ranged from 21% for alfalfa and one birdsfoot trefoil to virtually 100% for two of the sainfoins and three of the lespedezas. Generally, legumes with substantial levels of measurable tannins had slower protein degradation rates and greater estimated ruminal escapes than those without tannins. However, red clover (*Trifolium pratense* L.), which does not contain tannins (as measured by the radial diffusion assay) had a protein degradability comparable to several of the birdsfoot trefoils with low amounts of tannins. Some of the degradation rates observed for the 1990 samples were slightly negative; we interpreted this result to mean that these rates were essentially 0. However, it seemed unlikely that these forage proteins would be completely resistant to microbial degradation in the rumen. Therefore, most of the legumes from 1990 were retested in a Michaelis-Menten system which appears to give more reliable rates for very slowly degraded proteins. Degradation rates determined by this method were more rapid. The sainfoins and lespedezas which gave rates of less than .01/h and apparent escapes of 90 to 106% by the limited substrate approach yielded rates and escapes ranging from .03 to .05/h and 53 to 61% by the Michaelis-Menten method. Using the Michaelis-Menten approach, tannin containing forages again had slower rates and greater escapes; red clover and kura clover had the least degradable protein among the non-tannin legumes.

Conclusions

These data suggest that variability for ruminal protein degradation exists among forage legumes. A large part, but not all, of the variability observed in this set of legumes was associated with tannin concentration.

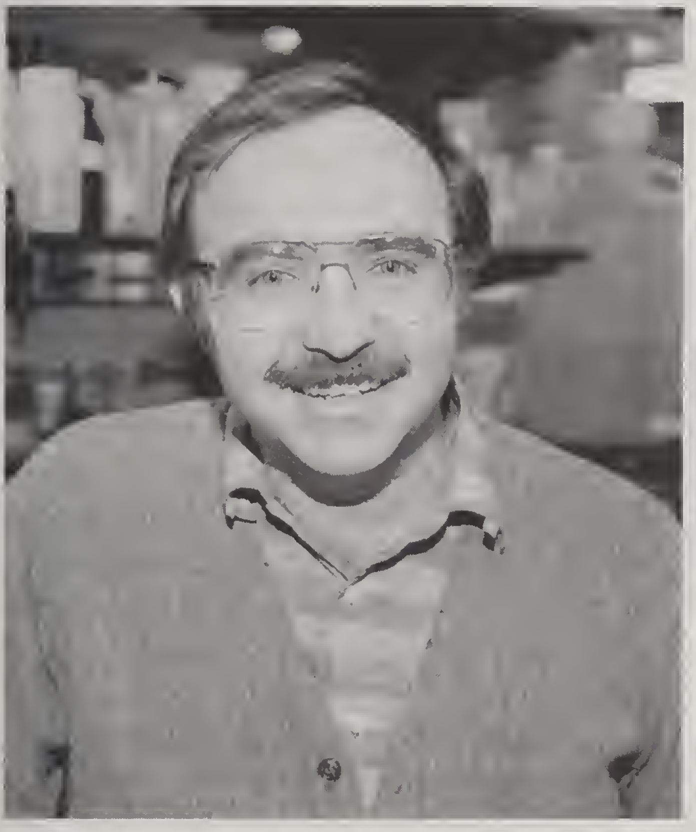
Table 1. Tannin levels and ruminal in vitro protein degradabilities of forage legumes.

	1989			1990				
Forage (cv.)	Tannin (g TAE/kg)	LS rate (/h)	LS escape (%)	Tannin (g TAE/kg)	LS rate (/h)	LS escape (%)	MM rate (/h)	MM escape (%)
Crownvetch (Monarch)	0	.295	16	—	—	—	—	—
White Clover (Ladino)	0	.286	16	—	—	—	.226	20
Alfalfa (Dart)	0	.269	17	0	.208	21	.205	22
Cicer Milkvetch (Monarch)	0	.261	17	0	.195	22	.266	18
Canada Milkvetch	0	.260	18	—	—	—	—	—
BFT (Norcen)	Tr	.254	18	Tr	.178	24	.266	18
BFT (WIT-II)	Tr	.244	18	—	—	—	—	—
BFT (Leo)	Tr	.243	19	Tr	.208	21	—	—
BFT (Carroll)	Tr	.225	19	—	—	—	—	—
Kura Clover (Rhizo)	0	.237	20	—	—	—	.159	26
BFT (Viking)	7.0	.192	22	8.5	.123	31	.176	24
Red Clover (Marathon)	0	.163	26	0	.135	29	.155	27
Sainfoin (Remont)	14.6	.061	46	22.1	.015	77	.070	43
L. pedunculatus (Maku)	17.3	.063	46	28.6	.023	69	.094	38
Sainfoin (Nova)	15.9	.046	53	27.0	-.002	102	.045	54
Sainfoin (Eski)	20.2	.044	55	29.5	-.002	100	.046	54
Lespedeza (AU Lotan)	18.4	.023	69	19.5	.018	74	.038	59
Lespedeza (AU Donnelly)	18.1	.015	77	12.1	.041	57	.045	56
Lespedeza (I-76)	26.3	.012	80	28.0	-.007	108	.051	53
Lespedeza (Serala)	26.3	.005	89	27.7	.005	90	.032	63
Lespedeza (Serala 76)	26.4	.004	90	29.4	-.006	106	.040	59
Lespedeza (Interstate)	28.2	.000	96	31.1	-.006	105	.034	61

LS = Limited substrate
MM = Integrated Michaelis-Menten
TAE = Tannic acid equivalents
Tr = Trace
BFT = Birdsfoot trefoil

EFFECT OF PROTEIN DEGRADABILITY ON MICROBIAL PROTEIN YIELD IN VITRO

G. A. BRODERICK and A. HRISTOV



Glen A. Broderick

Introduction

Argyle and Baldwin (J. Dairy Sci. 72:2017, 1989) reported an up to 4-fold increase in microbial growth over the NH_3 control when amino acid mixtures or tryptic digests of casein were added to in vitro media. This and other results suggested that the supply of peptides and amino acids, which would be related to ruminal protein degradation, might be a critical determinant of the amount of microbial protein supplied to the cow. When rates and extents of protein degradation were estimated in an in vitro system, microbial protein yields also were measured using $^{15}NH_3$ (Hristov and Broderick, 1990 USDFRC Res. Sum., p. 96). This summary relates the previously reported rates and extents of protein degradation to net microbial protein yields.

Materials and Methods

Methods used to conduct the incubations, including preparation of ruminal inoculum, incubation conditions, level of $^{15}NH_3$ addition, isolation of ruminal microbial pellets, measurement of ^{15}N enrichments in NH_3 and ruminal microbes, replication and statistical analysis, and computations of rates and extents of protein degradation are described in our earlier report (Hristov and Broderick, 1990 USDFRC Res. Sum., p. 96). Net microbial N (NMN) formation at the end of the incubation (6-h) was computed for each protein using the equations:

$$MN = (TS^{15}N / B^{15}N) \text{ X } TSN \quad (1)$$

$$NMN = MN_{prot.} - MN_{blank} \quad (2)$$

where MN is microbial N (mg/100 ml), $TS^{15}N$ and $B^{15}N$ are ^{15}N enrichments of total solids N and bacterial N, TSN is total solids N (mg/100 ml), and $MN_{prot.}$ and MN_{blank} are microbial N (mg/100 ml) in protein-added and blank vessels. Seven proteins with varying degradabilities were tested: Casein, solvent soybean meal (SSBM), expeller soybean meal (ESBM), low solubles fish meal (LSFM), high solubles fish meal (HSFM), corn gluten meal (CGM), and roasted soybeans (RSB).

Results and Discussion

Mean N metabolism data from the incubations are in Table 1. Extents of protein degradation after 6-h ranged from 14.6 (LSFM) to 97.0% (casein); degradation rates ranged from .026 to .569/h. These rates yielded estimated ruminal escapes ranging from 10 to 70%. Protein degradation was substantially underestimated from apparent NH_3 release because of extensive microbial uptake of NH_3 for growth; apparent NH_3 release for ESBM, CGM and RSB was almost nil (Table 1). Ratio of ^{15}N enrichment of microbial N at 6 h (product) to the average of ^{15}N enrichments of NH_3 at 0 h and 6 h (precursor) was used to compute proportions of microbial N coming from the NH_3 and non-NH_3 N (NAN) pools (Figure 1). Except for casein, proportions of microbial N from NH_3 and NAN were similar among incubations; microbial N incorporated from $^{15}NH_3$ ranged from 28% (casein) to 46% (blank, CGM and RSB). A strong preference of the microbes to use N from NAN rather than NH_3 is apparent despite NH_3 concentrations of 8.7 to 15.4 m*M*, well in excess of the "optimal" 3.6 m*M*.

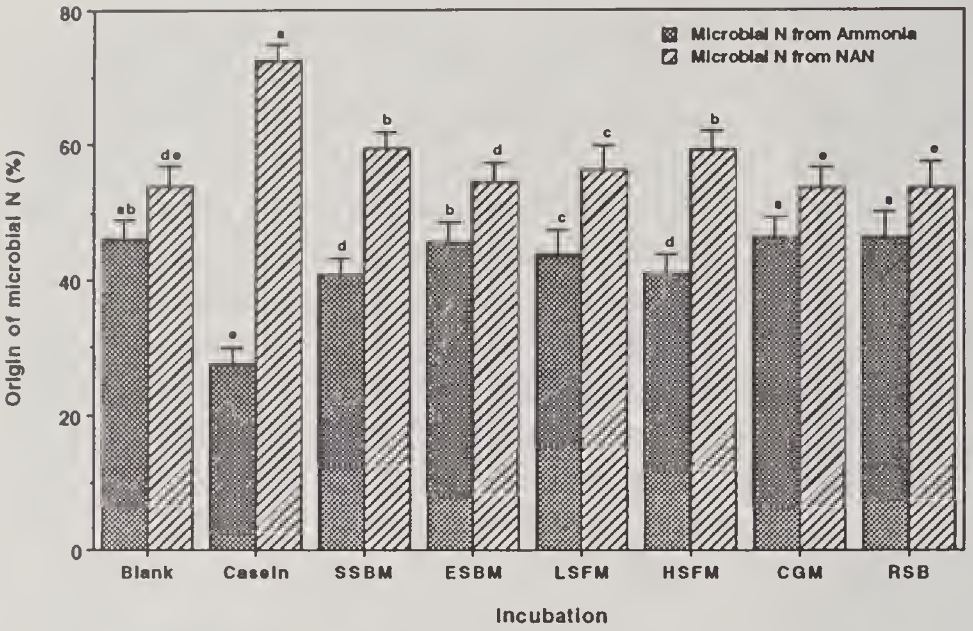


Figure 1. Proportion of N incorporated into microbial protein N originating from NH3 and NAN at the end of the incubation (means ± SD). Letters represent mean separations, within N source, determined by LSD (P < .05).

Actual ^{15}N enrichment of nascent microbial protein N probably was greater than observed enrichment due to dilution from unlabelled microbial N carried over from the inoculum. Our estimates of microbial N from NH_3, uncorrected for carryover, were low compared to reports of 50-78% for bacteria and 31-64% for protozoa and 42-61% for mixed ruminal organisms.

Regression of mean NMN yield from each incubation on extent of degradation at 6 h for the seven proteins (Figure 2) indicated a high correlation ($P < .001$; $r^2 = .780$); this suggested a stimulatory effect of peptides and free amino acids on microbial protein

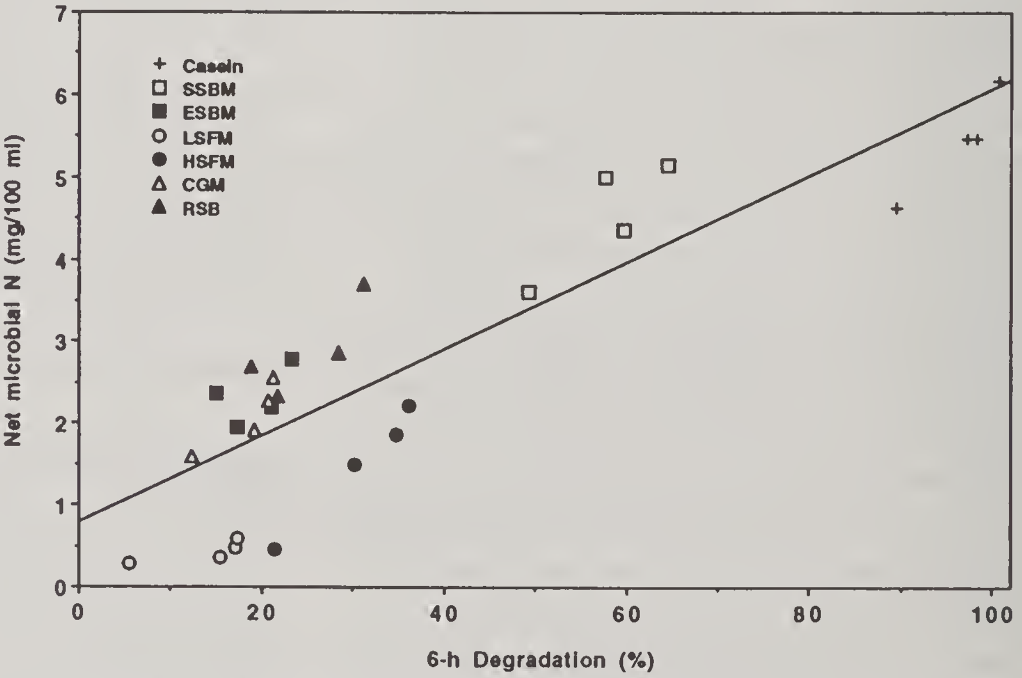


Figure 2. Regression of net synthesis of microbial protein N on extent of degradation at 6 h (the end of the incubation). Y = .053 X + .778 (r^2 = .780).

synthesis. Several workers, including Argyle and Baldwin (1989), found that free amino acids and acid or enzymatic hydrolysates of casein stimulated microbial growth even when NH_3 and VFA were not limiting. A similar response in bacterial N formation was reported by Stokes et al. (J. Dairy Sci. 74:860, 1991) in continuous culture studies with mixed ruminal organisms when degradable protein was added as peanut meal. Formation of NMN for the fish meals was lower than for the other five proteins: All eight values for LSFM and HSFM fell below the regression line (Figure 2). Oil levels of fish meals in our study were (DM basis) 6.4 (LSFM) and 9.9% (HSFM). An inhibitory effect of fish oil may account for suppression of NMN. Hussein et al. (J. Anim. Sci. 69:2123, 1991) found that, compared to soybean meal, feeding fish meal to provide oil concentrations equivalent to 5-6 times those used in our study depressed bacterial N flow from continuous culture fermentors. Relationship of NMN yield to degradation rate is shown in Figure 3. Microbial protein formation appeared to be a linear function of degradation rate for all proteins except casein ($P < .001$; $r^2 = .676$); mean NMN increased only from 4.6 (SSBM) to 5.5 (casein) mg N/100 ml as degradation rate increased from .148 to .569 /h. Intersection of the regressions at .14/h, approximately the degradation rate for SSBM, suggested that dietary protein with degradability more rapid than this rate would not increase microbial protein synthesis.

Summary and Conclusion

Ruminal in vitro protein degradation rates and estimated escapes for seven proteins ranged from .026 to .569/h and 10 to 70%, respectively. More rapid protein degradation gave rise to greater NAN incorporation and lower NH_3 incorporation. There was a strong linear relationship between extent of protein degradation at the end of the incubation and net microbial protein yield. However, degradation rates greater than .14/h did not appear to further increase microbial yield.

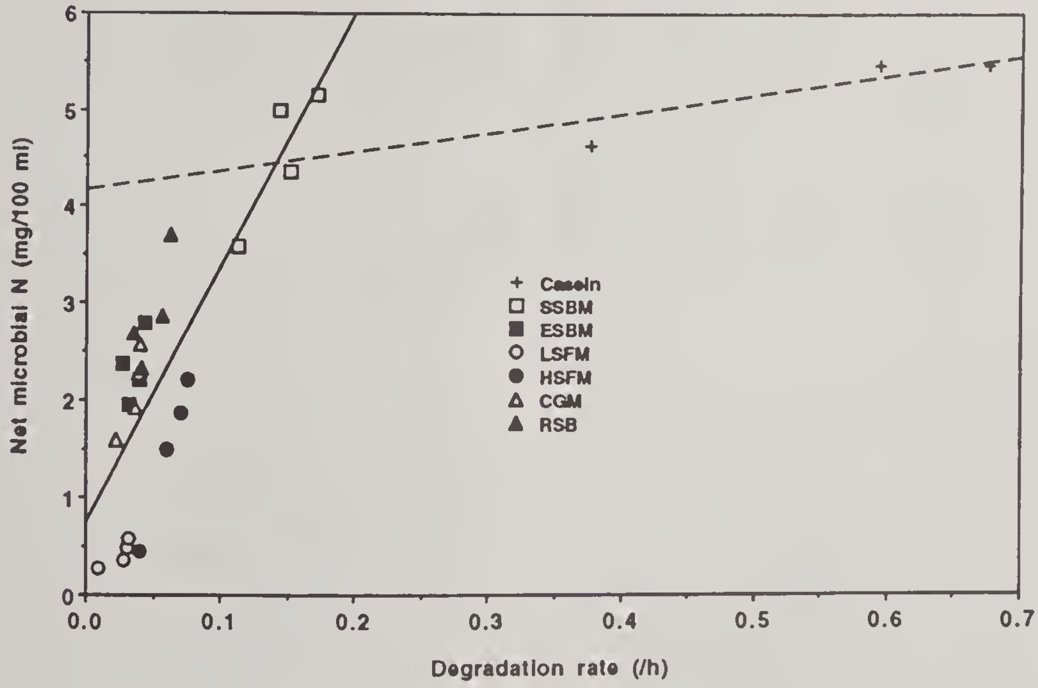


Figure 3. Regressions of net synthesis of microbial protein N on rates of protein degradation. Regressions are for all proteins except casein [———— ; $Y = 25.98\,X + .779$ ($r^2 = .676$)] and for SSBM and casein only [- - - - - - -; $Y = 1.97\,X + 4.18$ ($r^2 = .468$)].

TABLE 1. Protein metabolism data from in vitro incubations.[1]

Item	Blank	Casein	SSBM	ESBM	LSFM	HSFM	CGM	RSB	SEM
NH3 N, mg/100ml	8.70^{e}	15.35^{a}	11.42^{b}	8.76^{e}	10.08^{d}	11.03^{c}	8.89^{e}	8.99^{e}	.11
Apparent NH3 N release (A), mg/100ml	---	6.65^{a}	2.72^{b}	$.06^{e}$	1.39^{d}	2.33^{c}	$.19^{e}$	$.29^{e}$	.11
Total solid N, mg/100ml	24.73^{f}	28.50^{e}	33.05^{d}	35.78^{b}	34.22^{c}	33.86^{cd}	36.86^{a}	36.40^{ab}	.34
Total solid ^{15}N, atom % excess	$.455^{a}$	$.278^{f}$	$.336^{b}$	$.333^{bc}$	$.315^{d}$	$.303^{e}$	$.326^{c}$	$.335^{bc}$	.003
Bacterial ^{15}N, atom % excess	$.567^{a}$	$.312^{e}$	$.456^{d}$	$.540^{b}$	$.537^{b}$	$.481^{c}$	$.551^{ab}$	$.540^{b}$	.006
Microbial-N, mg/100ml	19.84^{f}	25.34^{a}	24.45^{b}	22.20^{cd}	20.28^{f}	21.44^{e}	21.94^{de}	22.75^{c}	.22
Net microbial N (B), mg/100ml	---	5.50^{a}	4.61^{b}	2.36^{cd}	$.44^{f}$	1.60^{e}	2.10^{de}	$.2.91^{c}$	.26
Degraded protein N (A + B), mg/100ml	---	12.16^{a}	7.33^{b}	2.42^{e}	1.83^{e}	3.93^{c}	2.29^{e}	3.20^{d}	.23
Degraded protein N, %	---	97.0^{a}	58.6^{b}	19.3^{e}	14.6^{e}	31.4^{c}	18.3^{e}	25.6^{d}	1.9
Degradation rate (k_d),/h	---	$.569^{a}$	$.148^{b}$	$.036^{c}$	$.026^{c}$	$.063^{bc}$	$.034^{c}$	$.050^{bc}$	.037
Estimated escape, %	---	10.0^{e}	29.1^{d}	62.9^{ab}	70.1^{a}	49.2^{c}	64.4^{ab}	55.3^{bc}	4.3

[a,b,c,d,e,f] Means within rows having different superscripts differ ($P < .05$).

[1] SSBM = Solvent soybean meal; ESBM = expeller soybean meal; LSFM = low solubles fish meal; HSFM = high solubles fish meal; CGM = corn gluten meal; RSB = roasted soybeans. Probabilities of significant treatment effects were $P < .001$ for each trait.

THE RESISTANCE OF PROLINE-CONTAINING PEPTIDES TO RUMINAL DEGRADATION *IN VITRO*

C.M. J. YANG and J.B. RUSSELL

Introduction

Much of the protein which is ingested by ruminants is deaminated by ruminal microorganisms. Some of this ammonia can be used as a nitrogen source for microbial growth, but the production rate of ammonia often exceeds the utilization rate. ^{15}N studies indicated as much as 25% of the protein can be lost from the rumen as ammonia and is subsequently converted to urinary urea. Many insoluble proteins are not completely degraded in the rumen, but these "escape" or "by-pass" supplements (fish meal, brewer's grains, etc.) are more expensive than forage proteins or soybean meal.

In the early 1980's, Brock et al. examined the effect of various proteinase inhibitors on ruminal protein degradation. Virtually all of the inhibitors tested had some effect, but only 5 mM methiolate, a general antimicrobial agent, gave more than 50% inhibition. Based on these results, it appeared that ruminal microorganisms produced a variety of different proteinases, and that it would be difficult to manipulate protein degradation at this step.

Because amino acids are generally present at low concentrations in ruminal fluid, it had been assumed that proteolysis was the rate limiting step in ruminal protein degradation and the role of peptidases was largely ignored. When Mangan added casein to the rumen, there was an increase in non-ammonia, non-protein nitrogen, and this observation indicated that peptides could accumulate. Some workers have suggested that peptide accumulation might be an artifact of casein, a rapidly degraded, non-feedstuff protein, but significant amounts of non-ammonia, non-protein, ninhydrin reactive material were also noted in the rumens of cattle fed soybean protein.

Materials and Methods

Mixed ruminal bacteria were incubated anaerobically with protein hydrolyzates or synthetic peptides. The amino acid composition of the residual peptides and free amino acids (96 h) was estimated by high pressure liquid chromatography (HPLC). Cell-free samples (25 ml) were hydrolyzed with HCl and derivitized with phenylisothiocyanate prior to being added to the HPLC column. This procedure gave the total amino acid composition of the samples. Peptides were then estimated by difference from samples which had been run through a C-18 Sep-Pak to remove peptides (Millipore Corporation, Milford, MA).

Results and Discussion

Mixed ruminal bacteria utilized an enzymatic digest of casein at a faster rate than an enzymatic digest of gelatin, but neither amino acid source was completely utilized even if the incubation period was as long as 96 h. Since the reaction of ninhydrin with the residual non-ammonia, non-protein nitrogen was more than 2-fold stronger when the samples were hydrolyzed with 6 N HCl, it appeared that much of the residual nitrogen was peptides. Approximately 66% of the non-ammonia, non-protein, ninhydrin-reactive material could not be recovered as amino acids, but there was a significant decrease in total amino acid nitrogen when the samples were pretreated with a C-18 Sep-Pak column to remove peptides. The resistant peptides had an abundance of proline, and subsequent incubations showed that synthetic dipeptides which contained proline were hydrolyzed slowly. Lysine appears to be the amino acid which is most apt to limit ruminant production. Dipeptides containing proline and lysine were hydrolyzed at least five-fold slower than lysine-alanine. Methionine, another potentially limiting amino acid, was also degraded at a slower rate (2.5-fold) when it was present as part of a proline dipeptide.

Conclusions

When Schwab et al. infused casein or amino acids into the abomasums of lactating dairy cattle, a combination of lysine and methionine gave 43% of the total increase in milk protein. Based on these results, it appeared that lysine and methionine were the most limiting amino acids. There have been attempts to protect lysine and methionine by capsules which are ruminally inert but soluble in the acidic abomasum. While this approach may have merit, problems can arise. If the capsule does not have some resistance to low pH, it can decompose in silage acid. If the capsule is too acid resistant, the lysine or methionine may be poorly absorbed from the intestines. Our experiments indicated that lysine and methionine dipeptides which contained proline were degraded slowly by bacteria in ruminal fluid. Because the initial rates of proline dipeptide degradation were lower than the rumen fluid dilution rate, it is likely that these peptides could escape ruminal degradation. The only exceptions were proline-alanine and proline valine. Based on this latter comparison, proline may provide better protection when it is present at the C-terminal end of the dipeptide. Further work is needed to examine the intestinal absorption of proline-containing peptides.

PHYLOGENY OF AMMONIA-PRODUCING RUMINAL BACTERIA, *PEPTOSTREPTOCOCCUS ANAEROBIUS, CLOSTRIDIUM STICKLANDII*, AND *CLOSTRIDIUM AMINOPHILUM*

B. PASTER, J.B. RUSSELL, C.M.J. YANG, J.M. CHOW, C.R. WOESE and R. TANNER

Introduction

The rumen is one of the best studied microbial habitats, and for many years it had been assumed that the predominant classes of ruminal microorganisms had been isolated. Much of the protein which enters the rumen is fermented, but Bladen et al. (1961) noted that most pure cultures of ruminal bacteria were unable to produce ammonia. Strains of *Bacteroides ruminicola, Megasphaera elsdenii, Selenomonas ruminantium, Eubacterium ruminantium* and *Butyrivibrio fibrisolvens* produced some ammonia, but less than 25% of the casein hydrolysate was converted to ammonia even though the incubation period was 96 h. Nonetheless, "on the basis of numbers of strains and amount of ammonia produced," Bladen et al. (1961) concluded that "*Bacteroides ruminicola* is usually the most important ammonia-producing bacterium in the rumen of mature cattle." All of these ammonia-producing bacteria fermented carbohydrate, and the isolation media contained carbohydrates and a low concentration of Trypticase.

Later work indicated that the $B_1$4 strain of *B. ruminicola*, the most active one examined by Bladen et al. (1961), had a specific activity of ammonia-production which was less than mixed cultures of ruminal microorganisms. Interspecies hydrogen transfer and methanogenesis increased the capacity of mixed ruminal bacteria to produce ammonia, but this effect could not explain the low activity of the pure cultures. Protozoa also produced some ammonia, but their activity was even lower than the bacteria, particularly when the amino acid source was soluble. The feed-additive, monensin, decreases ammonia production in vitro and in vivo, but the most active ammonia-producing ruminal bacteria were monensin-resistant.

Based on this earlier work, it appeared that very active amino acid-fermenting bacteria had not yet been isolated from the rumen. Enrichments which contained Trypticase as the sole nitrogen source and lactate, dulcitol, pectin or xylose as an energy source yielded strains of *M. elsdenii, S. ruminantium, B. ruminicola*, and *B. fibrisolvens*, respectively, but none of these bacteria had specific activities of ammonia production which were greater than the ones examined by Bladen et al. (1961). When the concentration of Trypticase was increased and other energy sources were omitted, a large gram-positive coccus was isolated (strain C), and this isolate had a specific activity of ammonia production which was nearly 20-fold greater than *B. ruminicola* B_14. Later isolations yielded two more gram-positive bacteria, a pleomorphic rod (strain F) and short rod (strain SR), which could not utilize carbohydrates as an energy source for growth. These recently isolated amino acid-fermenting bacteria have not been enumerated extensively, but they were found at significant numbers in the rumen. Their sensitivity to the feed additive, monensin, is consistent with the "amino acid sparing" effect of this antibiotic.

Materials and Methods

Ribosomal RNAs were isolated and partially purified using the procedure of Pace et al. as described by Paster and Dewhirst. Nearly complete 16S rRNA sequences were determined by a Sanger dideoxy chain termination technique in which seven primers complementary to universally conserved regions are elongated with reverse transcriptase. The procedure was modified to reduce termination bands. RNA sequence, sequence alignment, secondary structure, similarity matrix, and phylogenetic trees were analyzed by a Microsoft Quick BASIC program using an IBM PC-AT as previously described. The data base contained RNA sequences from approximately 350 different bacterial species. These sequences were obtained from previously published articles, unpublished sequences that were provided by other scientists, and unpublished work from our own laboratory. Only those regions that could be unambiguously aligned were used to construct similarity matrices. The Neighbor-Joining method of Saitou and Nei was used to construct phylogenetic trees.

Results and Discussion

Based on phenotypic criteria, the taxonomic position of the new isolates was uncertain. In this study, the 16S rRNA sequences of these isolates and related bacteria were determined to establish their phylogenetic positions. Sequences of strain C, strain F, and strain SR and reference strains of *Peptostreptococcus anaerobius, Clostridium sticklandii, Clostridium coccoides, Clostridium aminovalericum, Acetomaculum ruminis, Clostridium leptum, Clostridium lituseburense, Clostridium acidiurici, Clostridium barkeri*, were determined using a modified Sanger dideoxy chain termination method. Strain C, a large coccus purported to belong to the genus *Peptostreptococcus*, was closely related to *Peptostreptococcus anaerobius* with a sequence similarity of 99.6%. Strain SR, a heat-resistant, short rod, was closely related to *C. sticklandii* with a sequence similarity of 99.9%. However, strain F, a heat-resistant, pleomorphic rod, was only distantly related to some clostridial species and *Peptostreptococcus anaerobius*. Based on the sequence data, it was clear that strain F warranted a separate species designation. The closest known relative to strain F was *Clostridium coccoides* with only 90.6% similarity. Additional strains that are phenotypically similar to strain F were isolated in this study.

Conclusions

Based upon phenotypic and phylogenetic differences, we believe that strain F represents a new species of the genus Clostridium, for which we propose the name *Clostridium aminophilum.*

GLUCOSE TOXICITY AND THE INABILITY OF *BACTEROIDES RUMINICOLA* TO REGULATE GLUCOSE TRANSPORT

J.B. RUSSELL

Introduction

The rumen is an ideal habitat for the growth of anaerobic bacteria, and it approximates a continuous culture system. Food and water are ingested on a regular basis and this dilution expels undigested food and microorganisms from the rumen. Fermentation acids are absorbed across the rumen wall, and pH is regulated by salivary bicarbonate. Because the density of ruminal microorganisms is very high, the rumen usually operates as an energy-limited chemostat, but other nutrients can limit bacterial growth .

Microbial protein is the primary source of amino acids entering the small intestine, and the efficiency of rumen microbial protein production can have a profound impact on ruminant production. Food enters the rumen on a regular basis, but ^{15}N labeling experiments indicated that much of microbial mass (15 to 20%) can turn over before it leaves the rumen. Hespell and his colleagues showed that some ruminal bacteria are sensitive to even brief periods of energy starvation. A rapid loss in viability would decrease the flow of microbial protein from the rumen. *Bacteroides ruminicola* is a versatile bacterium that can account for as many as 19% of the cultivable bacteria from the rumen. Most strains of *B. ruminicola* digest starch, but their numbers are highest when forages are fed.

Materials and Methods

B. ruminicola was grown under standard anaerobic conditions. Viable cell numbers were estimated by the most probable number method. Cells were washed anaerobically and incubated with ^{14}C glucose to estimate glucose transport activity. Cell protein and carbohydrate were estimated by the Lowry and anthrone procedures, respectively. ATP was estimated with a luminometer using luciferase.

Results and Discussion

Ammonia-limited (3.5 mM ammonia) cultures of *B. ruminicola* $B_1 4$ had a high number of viable cells (> 10^9/ml), but only if the concentration of glucose was not too high (10 mM or less). When the glucose concentration was increased from 10 to 50 mM, there was a marked decrease in viability (10^5 fold or greater). Because there was little decline in pH and only a small increase in succinate and acetate as the glucose concentration was increased, it did not appear that endproducts were killing the cells. This conclusion was supported by the observation that re-inoculated cultures grew in the spent medium which had been supplemented with ammonia. Unlabeled rhamnose did not inhibit ^{14}C glucose uptake, and cultures which were selected with a low concentration of rhamnose tolerated high concentrations of glucose (50 mM). The glucose-resistant mutant transported glucose at a slower rate than the wild type, and the V_{max} of glucose transport was 4-fold lower. The wild-type stored much more polysaccharide than the glucose-resistant mutant, but it is not clear if polysaccharide accumulation *per se* is responsible for the glucose toxicity. These results indicated that *B. ruminicola* B_14 is unable to regulate glucose transport and utilization when growth is limited by ammonia.

Conclusions

The effect of nitrogen limitation on the growth and viability of bacteria has not been studied in a systematic fashion, but it is apparent that many organisms can tolerate nitrogen-limited conditions with an excess of carbohydrate. In the 1970's Neijessel and Tempest grew *Klebsiella aerogenes* in a nitrogen-limited chemostat with 167 mM glucose, and several species of ruminal bacteria have been grown in ammonia-limited chemostats with more than 20 mM hexose. Schaefer et al. used ammonia-limited continuous cultures to estimate the affinity of *B. ruminicola* B_14 for ammonia, but in this case the glucose concentration was only 12 mM. At this time, the impact of glucose on the growth of *B. ruminicola* in vivo is difficult to assess.

EFFECT OF MATURITY OF ALFALFA SILAGE ON MILK PRODUCTION AND RUMEN FERMENTATION OF MID-LACTATION COWS

C.F. LEE and L.D. SATTER

Introduction

This study is from the third year of a three year study to evaluate the effect of maturity of alfalfa on milk production and rumen fermentation.

Materials and Methods

Alfalfa was harvested under a 3-cut (approximately 10% flower) or a 4-cut (at early to mid-bud) system, and stored in bunker silos. The increased stress of 4 cuttings/year decreased stand persistence, and yields for the 3-cut and 4-cut systems in the third year after seeding were 3.2 and 2.8 tons/acre, respectively. A harsh winter and dry spring reduced yields for both treatments. Neutral detergent fiber and crude protein for the 3-cut and 4-cut treatments before ensiling were: 44.3 and 17.3 and 39.3 and 19.8 % DM, respectively.

Experiment 1. Forty-eight mid-lactation cows were randomly assigned to a 2 x 2 factorial design. Two dietary forage levels (55 and 75%), and two maturities of alfalfa silage (3-cut and 4-cut) were used. Cows were group-fed a total mixed ration once daily. The 55% forage diets contained: 55% alfalfa silage, 28% high moisture ear corn, 10% roasted soybeans, 4.6% soybean meal and 2.4% of a vitamin-mineral mixture. The 75% forage diets contained 75% alfalfa silage, 13.2% high moisture ear corn, 10% roasted soybeans and 1.8% vitamin-mineral supplement. The experiment consisted of a 2 week pretrial, 7 week treatment, and 2 week switchback period. Milk production, milk composition, dry matter intake, and body weight were measured. Data from weeks 2-7 of the treatment period were used for statistical analysis.

Experiment 2. Four rumen cannulated mid-lactation cows were used in a 4 x 4 Latin square design to study the effect of maturity of alfalfa silage on the characteristics of rumen fermentation. Diet treatments were the same as experiment 1. Each period consisted of two weeks with the first week as the adjustment period. Rumen contents were sampled for pH, volatile fatty acids (VFA) and ammonia measurements before the morning feeding and 2, 4, 6, 12, 18, and 24 hr. following feeding on days 11 and 13. On day 12, rumination behavior was observed every 5 minutes for 24 h. Rumen contents were manually emptied 3-4 h after feeding on day 14. The weight and volume of whole rumen contents were measured, and then returned to the cow assigned to the corresponding dietary treatment for the following period. This was done to reduce the time needed for dietary adjustment.

Results

Experiment 1. Chemical composition of the 3-cut and 4-cut alfalfa is in Table 1. Quality as measured by NDF and ADF for forages of these morphological maturities was exceptionally high. Fermentation characteristics of the 3-cut silage appeared slightly better than the 4-cut, having lower pH, acetate, butyrate and ammonia content. Both forage level and maturity significantly affected milk production (Table 2). The 4-cut alfalfa silage supported higher milk production than the 3-cut alfalfa. Maturity significantly decreased milk production only when alfalfa silage made up 75% of the diet.

Experiment 2. Treatment effects on milk and milk composition were minimal (Table 3). In terms of rumen fermentation, 3-cut alfalfa silage diets had significantly higher pH and lower total VFA concentration than the 4-cut groups. Forage level in the diet seemed to have more influence on the rumen fermentation than did maturity. There were no differences in eating times for the four treatments, but the 3-cut diets required significantly more time for rumination.

Conclusion

Increasing maturity of alfalfa from approximately mid-bud to 10% flower had a small and insignificant effect on milk production of mid-lactation cows fed forage and grain in a 55:45 ratio. A more pronounced effect of maturity was noted when forage constituted 75% of the diet DM.

Table 1. Chemical composition of alfalfa silage (Dry Basis).

Item	3-cut	4-cut
DM, %	40.2	35.8
NDF, %	43.8	37.7
ADF, %	37.3	30.2
ADL, %	7.5	5.7
CP, %	19.1	21.3
pH	4.61	4.82
Lactate, %	4.51	4.74
Lactate/Acetate	2.68	1.68
Butyrate, %	0.19	0.78
NH_3-N, % total N	8.0	10.3
NPN-N, % total N	46.6	38.4
ADIN-N, % total N	5.6	4.3

Table 2. Cow performance (Experiment 1).

	55%		75%		Significance		
Item	3-cut	4-cut	3-cut	4-cut	F	M	F x M
Milk, kg/d	25.5^{ab}	26.2^{a}	23.0^{c}	24.7^{b}	0.0001	0.018	NS
Milk fat, %	3.73	3.61	3.78	3.76	NS	NS	NS
Milk protein, %	3.22	3.28	3.19	3.20	NS	NS	NS
DMI, kg/d	22.4	19.9	21.5	19.7	-	-	-
BWC, kg/d	1.02^{a}	0.53^{c}	0.80^{ab}	0.74^{bc}	NS	0.002	0.015

DMI = Dry matter intake; BWC = Body weight change; F = Forage level; M = Maturity; F x M = Interaction of forage level and maturity.
Significance, $\alpha = 5\%$

Table 3. Characteristics of rumen fermentation (Experiment 2).

	55%		75%		Significance	
Item	3-cut	4-cut	3-cut	4-cut	F	M
Milk, kg/d	29.8^{a}	29.9^{a}	26.9^{b}	28.0^{ab}	0.002	NS
Milk fat, %	3.70	3.48	3.77	3.92	NS	NS
Milk protein, %	3.09^{ab}	3.17^{a}	3.04^{b}	3.02^{b}	0.003	NS
DMI, kg/d	28.1	23.8	21.2	23.0	NS	NS
pH	5.83^{ab}	5.72^{b}	6.08^{a}	5.98^{a}	0.002	NS
Acetate, molar %	57.0^{b}	56.9^{b}	59.0^{a}	58.4^{ab}	0.003	NS
Acetate/propionate	3.41^{ab}	3.36^{b}	3.73^{a}	3.71^{a}	0.002	NS
Total VFA, mM	124^{ab}	132^{a}	116^{b}	125^{ab}	0.011	0.008
NH_3-N, mg/dl	19.3	20.1	20.2	20.5	NS	NS
Eating, min/d	295	270	332	297	NS	NS
Ruminating, min/d	526	438	520	495	NS	0.016

Significance, $\alpha = 5\%$

EFFECT OF MATURITY OF ALFALFA SILAGE ON MILK PRODUCTION OF EARLY-LACTATION COWS

C.F. LEE and L.D. SATTER

Introduction

This study is from the second and third year of a three year study to evaluate the effect of alfalfa maturity on performance of cows during early-lactation. All data are combined from these two repeated trials.

Materials and Methods

Alfalfa was harvested under a 3-cut (approximately 5-10% flower) or a 4-cut (early to mid bud) system and stored in bunker silos. Three-cut alfalfa silage had higher dry matter, neutral-detergent fiber, and lower crude protein concentration than the 4-cut silage (Table 1).

A 2 x 2 factorial design was used in both years including two forage levels (45 and 65% of ration DM) and two maturities (3-cut and 4-cut). Total mixed rations containing 3-cut alfalfa silage had higher dry matter and fiber content than the 4-cut diets (Table 1). Cows immediately after calving were randomly assigned to one of these four diets. The experimental period consisted of a 2-week pretrial, a 7-week treatment, and a 2-week switch back period. Milk production, intake, body weight, milk composition, and total tract digestibility (TTD) were measured during the experimental period. Acid-detergent lignin was the internal marker for TTD measurements in the second year trial. Fecal samples were collected at the 3rd, 5th, and 7th week and composited for analyses. In the 3rd year, passage rates of 3-cut and 4-cut alfalfa silage were measured at the same time as TTD measurements during the 3rd to 7th week of the treatment period for each cow. Lanthanum (La), sprayed on the alfalfa silage and offered once to the cow, was the rate of passage marker for alfalfa. Ytterbium (Yb) was used as an external marker for TTD and was applied to the high moisture ear corn for a period of 10 days. La was dosed on the 8th day. Feces were collected during the last 3 days at 0 (before La dosing), 12, 18, 22, 26, 30, 34, 38, 42, 46, 52, 60, and 72 hr. The slope of the descending line of the log on the La concentration was considered equivalent to the rate of passage (Kp) of the forage.

Results

Based on pH, NH_3-N, butyrate, and lactate content, 3-cut alfalfa seemed to ensile better than the 4-cut silage (Table 1). For multiparous cows, forage level was the only factor significantly affecting milk production. The 45% forage diets supported higher milk production than the 65% forage diets. Milk fat and protein percentage were not affected. Maturity did not affect the milk yield and composition of multiparous cows. Digestibility was higher for the 4-cut diet. There was no difference in rate of passage of the 3-cut and 4-cut alfalfa silages for either multiparous or primiparous cows.

Maturity also did not affect milk production or milk composition for primiparous cows. The high forage diet tended to support lower total milk production, but milk fat percentage was higher. There was no difference in dry matter intake between treatments. Dry matter digestibility tended to be higher with the 4-cut diets. Cows fed low forage level diets had significantly higher dry matter digestibility.

Conclusion

The effect, in terms of milk production, of cutting alfalfa at 5-10% flower compared to early to midbloom was small or nonexistent in this two year study. Diet digestibility was higher for the 4-cut alfalfa.

Table 1. Chemical composition of alfalfa silage and diets of early-lactation trial (1991, 92).

	1991		1992	
AS[1]:	3-cut	4-cut	3-cut	4-cut
DM, %	38.6	35.7	39.5	35.5
NDF, %	46.7	41.4	45.1	37.5
CP, %	19.3	21.3	18.7	21.7
NPN-N, % TN[1]	47.8	44.9	46.8	42.7
NH$_3$-N, % TN[1]	9.3	9.7	8.0	11.0
pH	4.84	4.92	4.58	4.84
Lactate, %	4.29	3.86	5.16	5.08
Lac/Ace[1]	1.5	1.17	3.03	1.69
Butyrate, %	0.17	0.67	0.15	0.91

	1991				1992			
TMR[1]:	45-3x	45-4x	65-3x	65-4x	45-3x	45-4x	65-3x	65-4x
DM, %	53.8	50.6	47.6	43.9	51.2	48.0	45.2	42.1
NDF, %	30.2	28.1	36.3	33.2	30.2	26.9	35.2	30.4
CP, %	20.1	21.1	20.0	21.3	19.6	20.6	19.8	21.4

[1] AS: alfalfa silage; TMR: total mixed ration; TN: total nitrogen; Lac/Ace: lactate/acetate.

Table 2. Cow performance of early-lactation trials (1991, 92, n = 69).

	45-3x	45-4x	65-3x	65-4x	p-value Forage	 Maturity
I. Multiparous cows:						
Animal no.	10	11	10	9		
Milk yield, kg/d	43.2^{a}	44.1^{a}	35.2^{b}	36.7^{b}	0.0001***	0.22
Milk fat, %	3.64	3.56	3.82	3.65	0.13	0.15
Milk protein, %	2.84	2.82	2.89	2.84	0.37	0.41
Body wt change, kg/d	-0.19	0.08	-0.17	-0.14	0.52	0.35
DM intake, kg/d	23.7^{a}	23.7^{a}	21.3^{b}	20.6^{b}	0.0001***	0.44
NDF intake, kg/d	6.8^{ab}	6.2^{b}	7.4^{a}	6.4^{b}	0.037*	0.0001***
CP intake, kg/d	4.8^{ab}	5.0^{a}	4.3^{c}	4.4^{bc}	0.0001***	0.09
Rate of passage, %/hr	6.0	7.8	6.8	6.4	0.66	0.32
Apparent digestibility, %:						
DM	65.4^{b}	71.4^{a}	65.0^{b}	68.7^{ab}	0.21	0.0005***
NDF	42.0^{b}	52.0^{a}	42.7^{ab}	53.7^{a}	0.08	0.0005***
CP	69.0	73.6	69.9	72.2	0.84	0.008**
II. Primiparous cows:						
Animal no.	9	6	8	6		
Milk yield, kg/d	32.3	30.7	28.9	29.5	0.31	0.63
Milk fat, %	3.52^{ab}	3.30^{b}	3.70^{ab}	3.91^{a}	0.0048**	0.96
Milk protein, %	2.75	2.86	2.81	2.84	0.64	0.09
Body wt change, kg/d	0.11	0.34	-0.08	0.08	0.26	0.11
DM intake, kg/d	17.8	17.7	17.9	17.6	0.98	0.69
NDF intake, kg/d	5.1^{b}	4.6^{c}	6.3^{a}	5.4^{b}	0.0001***	0.0001***
CP intake, kg/d	3.6	3.7	3.6	3.8	0.71	0.045*
Rate of passage, %/hr	6.1	5.5	6.3	6.7	0.31	0.92
Apparent digestibility, %:						
DM	66.3^{ab}	69.9^{a}	63.0^{b}	64.8^{ab}	0.009**	0.08
NDF	44.0	50.0	45.0	48.0	0.80	0.049*
CP	68.7	72.4	67.1	68.2	0.048*	0.10

Note: Data of rate of passage came from 1992 study only.
^{abc}Means in same row with superscripts are significantly different, $P < 0.05$.

NUTRIENT BALANCE IN LACTATING DAIRY COWS FED FORAGE AND GRAIN IN VARYING RATIOS

T.R. DHIMAN, J. KLEINMANS, N.J. TESSMANN, H.D. RADLOFF and L.D. SATTER

Introduction

The most important source of variation in net energy value of feeds is caused by variation in digestibility. The objective of this study was to measure DM digestibility of diets containing forage and grain in varying proportions, and to compare net energy balance over an extended period of lactation when dietary NE_L was calculated from feed digestibility values or taken from NRC tables.

Materials and Methods

Forty-four multiparous and 43 primiparous cows at the time of parturition were placed on one of five treatments that had a forage content varying from 40-98.2% (DM basis) during the first 12 wk of lactation (early lactation). Forage content was increased during weeks 13-26 of lactation (mid lactation) to give diets containing 48.2 to 98.2% forage and again during weeks 27-44 (late lactation) to give diets containing 68.2 to 98.2% forage. Dry matter digestibility (DMD) was measured using Yb as a marker during early and late lactation.

Net energy balance for wks 1 to 36 of lactation was estimated using net energy intake minus milk energy output (Tyrrell and Reid, 1965. J. Dairy Sci. 48:1215) and energy spent for maintenance and live weight change (NRC, 1989). Net energy intake for each cow was calculated by multiplying energy concentration in the diet times DM intake of that cow. Net energy content of each diet was estimated by two methods: (a) using NRC (1989) NE_L values for individual dietary ingredients, and (b) using the equation NE_L (Mcal/kg DM) = .0307% DMD - .47 (Moe et al. 1972, J. Dairy Sci. 55:945). The NE_L values used for alfalfa silage, high moisture ear corn and soybean meal were 1.30, 1.91, and 1.94 Mcal/kg DM, respectively. Digestibility values measured during early and late lactation using Yb as a marker were used for calculating NE_L values for each of these periods, respectively. Midlactation NE_L values were calculated from a regression of early and late lactation values.

Results and Discussion

Net energy balances were calculated through wk 36 of lactation because a complete set of cows was not available after this time due to drying off. Cows in all treatments were in negative energy balance in early lactation. All groups attained energy balance at about six or seven weeks into lactation, with the exception of the all forage diet, and cows in this treatment were slower to achieve energy balance.

Two methods of calculating NE_L intake, using NRC (1989) NE_L values for individual feed ingredients or calculating NE_L intake from measured DMD, resulted in similar net balances of NE_L after 36 weeks of lactation (Table 1). The NRC estimates of NE_L value of feed stuffs used in this experiment seem reasonably accurate. The magnitude of error in calculating energy balance from NRC NE_L values was usually within 5% of NE_L intake.

Conclusions

Calculations of energy balance from input-output of NE_L by two methods of calculation were very similar and lends support to the accuracy of the NRC NE_L values for feedstuffs used in this experiment.

Table 1. Intake of net energy, output and balance in cows fed forage and grain in varying ratios[1], Mcal.

	Treatment							Treatment						
	1	2	3	4	5	SEM	P =	1	2	3	4	5	SEM	P =
	Multiparous cows							Primiparous cows						
NE_L intake[2]	8636^{a}	8523^{ab}	7906^{b}	7085^{c}	6094^{d}	229	.01	7371^{a}	6793^{b}	6494^{b}	5817^{c}	4726^{d}	146	.01
Milk energy output[3]	5354^{ab}	5433^{a}	4762^{bc}	4344^{c}	3692^{d}	210	.01	4541^{a}	4371^{ab}	4046^{bc}	3638^{c}	2807^{d}	156	.01
Maintenance requirement[4]	2543^{a}	2649^{a}	2510^{ab}	2510^{ab}	2380^{b}	52	.03	2318	2260	2237	2270	2149	46	.16
Body weight change requirement[4]	308	161	272	154	120	89	.5	431^{a}	336^{a}	364^{a}	193^{b}	199^{b}	47	.01
Apparent error in calculated NE_L balance, using NRC[2]	+431	+281	+363	+76	-97	162	.15	$+82^{a}$	-174^{b}	-152^{ab}	-284^{bc}	-428^{c}	86	.01
Apparent error in calculated NE_L balance, using DMD[5]	$+233^{ab}$	$+93^{b}$	$+660^{a}$	$+35^{b}$	-63^{b}	160	.03	$+393^{a}$	-303^{c}	$+106^{b}$	-404^{c}	-545^{c}	87	.01

[1] Sum of wk 1 through wk 36 of lactation. Balance was calculated by subtracting output (milk + maintenance + BW change) from intake.

[2] NE_L intake based on NRC (1989) NE_L values for individual dietary ingredients and DMI of individual cows.

[3] Calculated using the equation: Gross energy value (Kcal/lb of milk) = 40.72 (Fat %) + 22.65 (Protein %) + 102.77 (Tyrrell and Reid, 1965).

[4] Calculated using NRC (1989).

[5] NE_L intake calculated using measured dry matter digestibility (DMD) of the diet and the equation: NE_L, Mcal/kg DM = .0307 x DMD% - .047 (Moe et al., 1972).

INCREASING CARBOHYDRATE AVAILABILITY TO THE RUMEN MICROBES AND ITS EFFECT ON ANIMAL PERFORMANCE

T.R. DHIMAN and L.D. SATTER

Introduction

The high nonprotein N content and extensive degradation of alfalfa protein in the rumen results in poor utilization of dietary protein due to insufficient fermentable energy being available to trap the alfalfa N into microbial protein. The objective of the present experiment was to study the effect of increased carbohydrate availability from corn grain on digestion and lactation performance of cows fed diets containing alfalfa silage.

Materials and Methods

A preliminary in vitro digestibility study was used to screen five differently processed corn treatments for their fermentability, using VFA production and pH as indicators of fermentability. The five treatments in increasing order of fermentability were: dry shelled rolled corn, cracked corn, high moisture ear corn, flaked corn, and ground high moisture ear corn. The low, medium and high samples were selected for study in a lactation trial. Twenty-seven mid lactation cows were assigned to 3 groups according to milk yield. The experimental design was a 3 X 3 Latin square repeated 9 times. Each period was 3 wk. Data were collected during the last 2 wk of each period. Cows were fed diets containing 63.2% alfalfa silage along with either 35% dry shelled rolled corn (Trt 1) or high moisture ear corn (HMEC, Trt 2) or ground HMEC (Trt 3). HMEC was ground in a hammer mill using a screen size of 3/16", giving a finely ground product. Diets were balanced for minerals and vitamins and fed as a TMR once daily. The alfalfa silage had 48% DM, 20.1% CP, 46.2% NDF and 37.7% ADF (DM basis). Rolled corn and HMEC had 90.2, 69.9% and 9.61, 9.54% DM and CP, respectively. Daily feed intake and milk yield were recorded. Once a week milk samples from two consecutive milkings were analyzed for composition. Dry matter digestibility was determined using acid detergent lignin as an internal marker. Nine fecal grab samples were collected from each cow over a 40 h time period, and a composite sample was analyzed for acid detergent lignin. Blood samples were collected during the last day of each period at 5 h postfeeding from the tail vein or artery and analyzed for amino acids. Starch concentration was determined in the composite fecal sample of each cow.

Results and Discussion

Results are summarized in Table 1. Dry matter intake was highest with rolled corn, causing intake of estimated NE_L and protein also to be highest for this group. There was a 6-unit increase in DM digestibility when corn was fed as HMEC compared with rolled corn. The DM digestibility was further improved when the same HMEC was fed in ground form. Cows fed rolled corn excreted 1.7 kg/d starch in the feces compared with .5 and .07 kg/d for the HMEC and ground HMEC treatments.

There were no treatment differences for milk yield and composition; however, FCM was higher with HMEC because of a slight reduction in fat % in cows fed ground HMEC. Because of lower DMI and a slight increase in FCM, the feed efficiency was higher with HMEC and ground HMEC compared to rolled corn. There was a tendency for higher blood plasma branched-chain amino acids with HMEC and ground HMEC, indicating that protein supply to the animal may have been improved due to increased microbial protein synthesis in the rumen.

Conclusion

Feeding corn in the form of HMEC improved the utilization of starch by the cow. Grinding the HMEC further improved starch utilization.

The small benefit from grinding HMEC might be of greater value in very high forage diets. A longer term study is needed to determine if the fine grinding will cause some partitioning of energy away from milk fat to body fat.

Table 1. Nutrient intake, milk yield, milk composition and feed efficiency in cows fed three different forms of corn in alfalfa silage based diets.

	Treatment			
Item	1 (Rolled corn)	2 (HMEC)	3 (Ground HMEC)	SEM
DMI, kg/d	23.8^{a}	21.7^{b}	21.7^{b}	.2
NE$_L$,[1] Mcal/d	35.6^{a}	33.1^{b}	33.1^{b}	.3
Protein intake, kg/d	3.79^{a}	3.47^{b}	3.48^{b}	.04
Undegraded intake protein,[1] kg/d	1.17^{a}	1.07^{b}	1.07^{b}	.01
Dry matter digestibility,[2] %	58.2^{c}	63.8^{b}	66.5^{a}	.5
Starch concentration in feces				
g glucose/kg DM feces	133.2^{a}	43.1^{b}	5.1^{c}	4.3
Starch excreted in feces,[3] kg/d	1.68^{a}	.54^{b}	.07^{c}	.06
Milk, kg/d	25.9	26.5	26.1	.3
3.5% FCM, kg/d	25.5^{b}	26.2^{a}	25.5^{b}	.3
Fat, %	3.41	3.44	3.36	.04
Protein, %	3.04	3.05	3.04	.01
Fat yield, kg/d	.88^{b}	.91^{a}	.88^{b}	.01
Protein yield, kg/d	.78	.81	.79	.01
Lactose, %	4.76	4.77	4.75	.01
SNF, %	8.56	8.57	8.53	.02
Feed efficiency, kg FCM/kg DMI	1.076^{b}	1.221^{a}	1.181^{a}	.016
Blood plasma branched-chain				
AA, nM/ml	209^{b}	233^{a}	221^{ab}	8

^{ab}Means in the same row with different superscripts differ (*P* < .01).

[1] Calculated using NRC (1989). NE$_L$ values used for alfalfa silage, rolled corn, and HMEC were 1.35, 1.84, and 1.91, respectively.

[2] Measured using acid detergent lignin as a marker.

[3] Calculated using DMI and dry matter digestibility values of individual cows.

AMOUNT OF HEAT NEEDED TO OPTIMIZE PROTEIN UTILIZATION IN COTTONSEED BY RUMINANTS

JIH-TAY HSU, T.R. DHIMAN and L.D. SATTER

Introduction

The objective of this study was to identify the optimum heating conditions for cottonseed to improve its value as a protein supplement for lactating dairy cows.

Methods and Procedures

Part A. Linted cottonseed was roasted in a Jet-Pro roaster to give a variety of heat exposures and, in some cases, was steeped or conditioned for an additional 30 minutes. The following measurements were made: protein dispersibility index (PDI), a measure of protein solubility in water; undegraded intake protein (UIP), or 'by-pass' protein; available lysine, measured by the dinitroflorobenzene technique (AOAC, 1984); and postruminal available lysine, the amount of lysine reaching the small intestine. ***Part B.*** Linted cottonseed, heated to various degrees, was placed in Dacron bags and incubated in situ up to 48 hrs to estimate UIP. UIP was estimated using various ruminal dilution rates. ***Part C.*** Delinted cottonseed, heated to various degrees, was fed to Holstein heifers in a 5 x 5 Latin square experiment. The concentration of branched chain amino acids was measured in blood taken from the tail vein or artery. Since branched chain amino acids are not metabolized by the liver, their concentration in the extrahepatic circulation can reflect protein uptake from the small intestine. ***Part D.*** Milk production response to feeding of delinted cottonseed was measured in ten early lactation Holsteins in two 5 x 5 Latin squares. The same diets fed to heifers in Part C were used for this experiment.

Results and Discussion

Part A. The cottonseed treatments and results are shown in Table 1. PDI values decreased with heat treatment, and UIP increased. Available lysine decreased sharply at temperatures above 150°C. Maximum postruminal available lysine was achieved with temperatures between 141°C-30 min steep and 150°C-30 min steep. Similar results, not reported here, were obtained with delinted cottonseed.

Part B. Results from the Dacron bag study are in Table 2. The k_d values represent the fraction of protein disappearing from the Dacron bag every hour. These rates for rumen digestion were used to calculate estimates of UIP. The amount of UIP in the heated cottonseed samples was about 2 to 4 times greater than in the unheated control samples. Heating to at least 141°C-30 min significantly increased UIP. Higher temperatures further increased the amount of UIP, but the bulk of benefit was achieved by the 141° to 146°C heat exposure. Similar results, not reported here, were obtained with delinted cottonseed. This method provides information about protein degradability but does not give any information about the amount of protein that is irreversibly lost or damaged by excessive heat treatment.

Part C. Heating of cottonseed increased the concentration of branched chain amino acids in blood plasma, with the highest concentration being achieved with the two highest temperatures, although there was no statistical difference ($P < .05$) between treatments 146°C-30 min and 166°C-30 min (Table 3). Since the branched chain amino acids are not very susceptible to heat damage, one would not expect to see reduced absorption of these amino acids with the temperatures used in this study. There was a tendency ($P < .18$) for the essential amino acids to increase with increased treatment temperatures, but as expected, the increase was small. The same trend would be expected for total amino acids.

The concentration of lysine remained constant, except for the highest temperature treatment. With this treatment, lysine concentration in blood plasma tended to decrease, an indication perhaps that lysine availability was reduced with the highest treatment temperature.

This type of study reveals little about the *amount* of by-pass protein or postruminal available protein, but it can indicate the relative ability of treatment to improve protein nutrition of the animal. Based on this experiment, it appears that the optimum treatment was between 146°C-30 min and 156°C-30 min.

Part D. The results of this experiment are in Table 4. The highest milk yield was obtained with the cottonseed heated to 146°C and steeped for 30 minutes. This was the heat exposure found to be optimum for soybeans in our earlier work. This type of experimental design is suitable for identifying the best treatment, but it is not a good design for determining the actual milk production response that is likely to be achieved with feeding of heated cottonseed. Our experience with feeding of roasted soybeans is that it takes several weeks for the full response in milk production to become evident. The short three-week periods used in this experiment probably did not allow full expression of potential benefit from heat treating of cottonseed.

Conclusions

The optimum temperature for heat treating cottonseed for the purpose of improving protein utilization by ruminants is to bring the cottonseed to 146°C and then to steep or condition for 30 minutes without cooling.

Table 1. Results from Part A.

Treatment	PDI[1]	UIP[2]	Available lysine	Postruminal available lysine
	- - - - - - - %	- - - - - - -	- - - - - mg/g	nitrogen - - - - - -
Unheated control	93.6^{a}	23.7^{j}	194^{a}	47^{c}
134°C-0 min	87.4^{b}	28.7^{i}	193^{a}	56^{ab}
134°C-30 min	86.5^{b}	29.8^{hi}	182^{abc}	55^{ab}
141°C-0 min	77.7^{c}	31.7^{ghi}	186^{ab}	60^{a}
141°C-30 min	76.4^{c}	32.2^{gh}	179^{abcd}	57^{ab}
150°C-0 min	60.4^{d}	32.9^{fg}	176^{abcd}	58^{ab}
150°C-30 min	60.1^{d}	33.0^{fg}	185^{ab}	61^{a}
155°C-0 min	51.1^{e}	34.0^{fg}	167^{bcd}	57^{ab}
155°C-30 min	45.6^{f}	35.8^{ef}	158^{de}	57^{ab}
159°C-0 min	43.6^{g}	34.6^{efg}	159^{cde}	56^{ab}
159°C-30 min	32.5^{h}	37.1^{e}	140^{e}	52^{bc}
176°C-0 min	22.7^{i}	43.3^{d}	73^{f}	31^{d}
176°C-30 min	16.1^{j}	52.0^{c}	43^{g}	22^{e}
210°C-0 min	15.3^{j}	65.6^{b}	8^{h}	5^{f}
210°C-30 min	16.5^{j}	77.4^{a}	2^{h}	1^{f}
SE	0.46	1.06	7.9	2.5

[abcdefghij] Means in the same column not sharing a common superscript are different ($P < .05$).

[1] Modified protein dispersibility index (Nitrogen soluble in .02N NaOH solution).

[2] Undegraded intake protein, or by-pass protein.

Table 2. In situ protein degradation rate (k_d h^{-1}) and estimated undegraded intake protein (UIP; % of CP) of linted cottonseed using different dilution rates.

Treatment	kd	UIP .04h^{-1}	UIP .06h^{-1}	UIP .07h^{-1}
Unheated control	.23^{a}	9.0^{c}	12.4^{c}	13.9^{c}
141°C-30 min	.11^{ab}	17.5^{bc}	23.0^{b}	25.3^{b}
155°C-30 min	.08^{b}	22.0^{b}	28.2^{b}	30.7^{b}
159°C-30 min	.06^{b}	26.3^{b}	32.9^{b}	35.5^{b}
176°C-30 min	.03^{b}	38.7^{a}	44.8^{a}	46.9^{a}
SE	.036	2.93	2.92	2.90

^{abcd}Means in the same column without the same superscript differ ($P < .05$).

Table 3. Concentration of amino acids in blood plasma of cows fed one of five cottonseed treatments.

Treatment	Branched-chain Amino acids	Essential Amino acids	Total Amino acids	Lysine
		n-mole/ml plasma		
Unheated control	731^{bc}	1337	3115	136
136°C-30 min	705^{c}	1288	2999	130
146°C-30 min	771^{ab}	1386	3154	138
156°C-30 min	783^{a}	1411	3222	133
166°C-30 min	783^{a}	1388	3172	120
SE	20.7	37.9	74.0	6.1

^{abc}Means in the same column not sharing a common superscript are statistically significant ($P < .05$).

Table 4. Milk production and milk composition of cows fed cottonseed treatments.

	Cottonseed treatment				
Measurement	Unheated control	136°C 30 min	146°C 30 min	156°C 30 min	166°C 30 min
Dry matter intake, lb/day	25.2	25.4	25.9	25.8	24.6
Milk, lb/day	36.9^{b}	36.7^{b}	38.2^{a}	37.0^{ab}	35.8^{bc}
3.5% fat corrected milk, lb/day	35.7	35.6	37.0	36.0	35.9
Milkfat, %	3.31	3.32	3.32	3.32	3.53
Protein, %	2.87^{b}	3.01^{a}	2.94^{ab}	2.96^{a}	2.94^{ab}
Fat yield, lb/day	1.22	1.22	1.27	1.23	1.26
Protein yield, lb/day	1.06^{b}	1.09^{ab}	1.12^{a}	1.09^{ab}	1.05^{b}
Lactose, %	4.74	4.75	4.76	4.71	4.71
Solids not fat, %	8.38^{b}	8.52^{a}	8.46^{ab}	8.43^{b}	8.41^{b}

^{abc}Means in the same row not sharing a common superscript are statistically significant ($P <.05$).

MILK PRODUCTION OF DAIRY COWS FED FORAGE AS EITHER ALFALFA SILAGE OR ALFALFA HAY

G. A. BRODERICK

Introduction

Typical dairy rations formulated from alfalfa forages contain substantial amounts of degradable protein. Evidence from in vitro (Broderick et al., J. Dairy Sci. 75:2440, 1992) and in vivo (King et al., J. Dairy Sci. 72(Suppl. 1):554, 1989) studies indicated that alfalfa hay (AH) may have greater amounts of undegraded intake protein (UIP) than alfalfa silage (AS). Increased UIP supply with feeding alfalfa as hay versus silage may result in greater milk yield. A study was conducted with lactating cows fed diets in which all of the forage was from either AS or AH. The objectives of the study were to determine whether: 1) there were differences in milk production due to feeding forage as either AS or AH, and 2) milk production response to resistant protein, fed as fish meal (FM), differed when feeding AS or AH.

Materials and Methods

Second-crop alfalfa was cut on 6/25 and 6/26/91. The AS was wilted to about 40% DM, chopped and stored in an upright silo; AH was harvested as small rectangular bales from alternate wind-rows in the same fields as the silage. Mean composition of the AS and AH forages were, respectively (DM basis), 21.2 and 19.7% CP, 35 and 35% NDF, and 27 and 26% ADF. The AS contained 49.4% NPN (total N basis). Twenty multiparous cows, 38 DIM, were blocked into five groups of four cows each with nearly equal stage of lactation and assigned randomly to balanced 4 X 4 Latin squares. Four of the cows had permanent ruminal cannulae and were used for ruminal sampling on the last day of each period. Diets were fed for 3-wk periods (total 12 wk) before switching; production and intake data were analyzed from the 2nd and 3rd wk of each period. The four diets fed in the Latin squares as TMR were (DM basis): 1) AS, 69% AS plus 30% high moisture corn (HMC); 2) AH, 70% AH plus 29% HMC; 3) AS + FM, 69% AS, 27% HMC plus 3% FM; and 4) AH + FM, 70% AH, 26% HMC plus 3% FM. Diets were supplemented with 1.3% minerals and vitamins; all four diets contained about 1.6 Mcal NE_L/kg DM. The CP contents of the diets were (DM basis): 1) 17.3%, 2) 16.3%, 3) 19.1%, and 4) 18.0%.

Results and Discussion

Intake of DM was lower on the AS diet than the other three diets (Table 1). Despite lower DMI on AS, production of milk and all milk components was similar to that on AH; lower DMI was reflected in a BW loss on AS. The milk production response to FM supplementation of these two forages is interesting. Adding FM, an effective UIP source, to the AS diet significantly increased yield of milk and all milk components except fat; protein yield was increased about 80 g/day (Table 1). However, FM supplementation of the AH diet did not increase yield of milk or any milk component (Table 1). Although yield was similar on unsupplemented AS and AH, calculations based on the NRC system suggested that production was limited by different nutrients: absorbed protein limited milk yield on AS while NE_L supply limited production on AH. That UIP supplementation of AS resulted in significantly greater yield of milk and protein than on AH suggested that the silage diet was, as expected, a poorer source of UIP, despite being about 1% higher in CP. Ruminal ammonia and total amino acid concentrations also were significantly lower on the two diets containing AH (Table 2), indicating that degradability of protein in AH was lower than that in AS. When the absorbed protein inadequacy was corrected by feeding FM with AS, milk yield was increased to the limit determined by NE_L supply. These results suggested that alfalfa forage fed as silage served as a better energy source than hay. Nelson and Satter (1988 USDFRC Res. Sum., p. 81) observed greater milk production with AS versus AH when feeding diets which were probably limiting in energy supply. Milk and blood urea concentrations were higher with FM supplementation but were not influenced by forage source; blood glucose was not altered by diet (Table 1).

Summary and Conclusion

Results from this trial indicated that milk production was comparable on diets containing about 70% alfalfa, whether the forage was fed as hay or silage. However, supplementation with a UIP source resulted in significantly greater yield of milk and milk components, especially protein, on AS, but not on AH. This suggested that AS was a poorer source of absorbed protein but a better energy source than AH. The probable UIP advantage of AH over AS does not appear to result in greater milk production.

Table 1. Effect of forage source and fish meal on DMI, BW gain, production of milk and milk components, and concentrations of milk urea and plasma urea and glucose.[1]

Item	AS	AH	AS + FM	AH + FM	$P > F$[2]
DMI, kg/d	22.5[b]	23.3[a]	23.3[a]	23.8[a]	.045
BW gain, kg/d	-.40[b]	.39[a]	.20[a]	.52[a]	.003
Milk, kg/d	35.9[b]	35.7[b]	37.2[a]	36.3[ab]	.015
3.5% FCM, kg/d	34.9[b]	33.5[b]	36.3[a]	34.4[b]	.029
Fat, %	3.34	3.14	3.36	3.17	.582
Fat, kg/d	1.19	1.12	1.25	1.15	.074
Protein, %	3.02[b]	3.08[a]	3.12[a]	3.08[a]	.002
Protein, kg/d	1.08[c]	1.09[bc]	1.16[a]	1.11[b]	<.001
Lactose, %	4.79	4.81	4.73	4.78	.134
Lactose, kg/d	1.72	1.73	1.76	1.74	.093
SNF, %	8.56	8.64	8.60	8.61	.073
SNF, kg/d	3.07[b]	3.08[b]	3.20[a]	31.3[ab]	.006
Milk urea, m*M*	5.8[b]	5.8[b]	6.8[a]	6.8[a]	<.001
Plasma urea, m*M*	7.3[b]	6.8[b]	8.4[a]	8.5[a]	<.001
Plasma glucose, mg/dl	77	81	78	78	.086

[a,b,c] Means within trial having different superscripts differ ($P < .05$).
[1] BW = Body weight; AS = alfalfa silage; AH = alfalfa hay; FM = fish meal.
[2] Probability of a significant dietary treatment effect.

Table 2. Effect of forage source and fish meal on pH and concentration of ammonia and total amino acids in ruminal fluid.[1]

Item	AS	AH	AS + FM	AH + FM	$P > F$[2]
pH	6.07	6.01	6.11	6.04	.094
Ammonia, m*M*	15.8[a]	9.8[b]	16.7[a]	10.4[b]	.009
Total amino acids, m*M*	3.0[a]	1.2[b]	2.4[a]	1.0[b]	.047

[a,b] Means having different superscripts differ ($P < .05$).
[1] AS = Alfalfa silage; AH = alfalfa hay; FM = fish meal.
[2] Probability of a significant dietary treatment effect.

EFFECT OF STEAM HEATING ALFALFA HAY ON UTILIZATION BY LACTATING DAIRY COWS

G. A. BRODERICK, J. H. YANG and R. G. KOEGEL

Introduction

Alfalfa forage is valuable for feeding dairy cattle because of its high protein content. However, alfalfa protein often is utilized poorly by ruminants because of extensive degradation in the rumen. Heat treatment of oil seeds enhances protein resistance to ruminal degradation and improves animal performance. Heating alfalfa also may improve protein utilization. Replacing soybean meal protein with protein from alfalfa heat processed by dehydration reduced ruminal branched-chain VFA, indicating reduced ruminal degradation, and increased plasma branched-chain AA, indicating increased intestinal AA absorption (Price et al., J. Dairy Sci. 71:727, 1988). We have in vitro evidence that steam heating alfalfa hay reduced ruminal protein degradation (Yang et al., 1989 USDFRC Res. Sum., p. 69). The objective of this study was to determine if steam treatment of alfalfa hay would improve performance when it was fed to lactating cows: 1) with hay as all of the forage (Trial 1), or 2) with hay as a portion of the forage when the balance of the dietary forage came from alfalfa silage (Trial 2).

Materials and Methods

Field-dried, second-cutting alfalfa hay was harvested as small rectangular bales at late-bud to early bloom stages. A portion was set aside as unheated control (U), and a portion was heat treated (H), 12 bales at a time, in a large soil autoclave. Temperature in the autoclave was held to a maximum of 110°C by controlling steam pressure. Temperature readings from thermocouples located in three bales in each batch indicated that 45 min was required to reach an average of 100°C; mean heating time at 100 to 110°C was 47 min (mean total time in the autoclave = 92 min). Bale core samples were assayed chemically and for ruminal protein degradability using our inhibitor in vitro system. Hays U and H contained, respectively, 18.4 and 18.4% CP and 43 and 53% NDF (DM basis), 4.6 and 15.3% ADIN (total N basis), 29 and 50% net ruminal protein escape (total escape minus ADIN-CP).

In Trial 1, 14 multiparous cows, 81 DIM, were blocked into two groups of nearly equal production and stage of lactation and used in a switchback lactation study. Two cows with permanent ruminal cannulae were assigned to each group and sampled on the last day of each period. The trial was divided into four 2-wk periods. During periods 1 and 4, all cows were fed only U as forage. Periods 2 and 3 were the switchback (2 X 2 Latin square) part of the trial. Milk production and intake data from the 2nd wk of each period were analyzed statistically. Diets contained (DM basis) 81% of either alfalfa hay U or H, 18% cracked corn, .7% urea, plus vitamins and minerals; hays were chopped (6.0 cm) and fed with other ingredients as a TMR. Apparent digestibilities were estimated from fecal grab samples using indigestible ADF as an internal marker.

In Trial 2, 16 multiparous cows, 36 DIM, were blocked into four groups of four cows each with nearly equal stage of lactation and production and assigned randomly to four balanced 4 X 4 Latin squares. Four other cows at low production (c. 20 kg/d of milk) with permanent ruminal cannulae were used for ruminal sampling on the last day of each period. Diets also were fed for 2-wk periods before switching and production and intake data analyzed were from the 2nd wk of each period. The four diets fed in the Latin squares as TMR were (DM basis): 1) control (C), 76% wilted alfalfa silage (51% DM) plus 23% high moisture corn (HMC); 2) 76% alfalfa silage, 14% HMC, plus 9 % soybean meal (SBM); 3) 52% alfalfa silage, 23% HMC, plus 24% U (U); and 4) 43% alfalfa silage, 32% HMC, plus 24% H (H). The amount of CP from SBM in 2) equaled CP in U and H in 3) and 4). The HMC content of 4) was increased to equalize NE_L with the other diets based on computed NE_L contents of U and H. The CP contents of the diets were (DM basis): 1) 20.1, 2) 23.2, 3) 18.0, and 4) 17.1.

Results and Discussion

In Trial 1, apparent digestibility of ADF was unaltered but that of NDF and ADIN were substantially increased in diet H (Table 1). Intake of DM, milk concentrations of fat and lactose, and yields of FCM and fat were unaffected by hay source (Table 2). However, yields of milk, protein, lactose, and SNF, and milk concentrations of protein and SNF all were reduced significantly by feeding H (Table 2). Ruminal ammonia and branched-chain VFA, "isovalerate" (isobutyrate plus 2-methylbutyrate) and isobutyrate (Table 2), also were reduced with H (Table 2). Reduced ruminal ammonia and branched-chain VFA are related to decreased protein degradability in H (Table 2). Butyrate was lower but there were no differences in ruminal pH, acetate or propionate (Table 2).

In Trial 2, despite adding more HMC, replacing one-third of dietary alfalfa silage with H reduced apparent DM and OM digestibility about 2 percentage units (Table 1). Apparent digestibility of NDF and ADF generally were lower in diets containing U and H, and that of ADIN was lowest on diet C, intermediate on diets SBM and U, and greatest on diet H (Table 1). Apparent digestibility of ADIN formed by heating alfalfa hay was about 40% in both trials. Intake averaged about 1.7 kg/d of DM more on diet H than on diets C and U; DMI was intermediate on the SBM diet (Table 2). Increased intake on diet H was reflected in greater production of milk, lactose, and SNF and protein than on diets C and U; production was intermediate on the SBM diet. Diet had no effect on yields of FCM or fat or on concentration of any milk component. Both ruminal pH and ammonia concentration were lowest on diet H; ruminal ammonia was intermediate on diets C and U (Table 5). As with ammonia, ruminal concentrations of the branched-chain VFA, isobutyrate and 2-methylbutyrate plus isovalerate were lowest with diet H, intermediate on diets C and U, and highest on the SBM diet (Table 2). Ruminal concentrations of ammonia and branched-chain VFA followed milk and plasma urea concentrations and apparent N digestibility and were in proportion to dietary CP levels. Diet had no effect on BW change or total ruminal VFA in either trial.

Summary and Conclusion

Heat treating alfalfa hay with steam for 47 min at 100 to 110°C reduced ruminal protein degradability but also reduced energy digestibility. Feeding this hay as the only forage decreased milk production in cows fed diets with 81% alfalfa. However, feeding the steam heated hay to replace unheated hay or alfalfa silage at 24% of dietary DM increased DMI and production of milk and milk components when its reduced energy content was compensated for by feeding more concentrate. Milk production in cows fed the heated hay diet, which had 17% CP, was comparable with that on the SBM diet containing 23% CP. Alfalfa hay appears to be more sensitive to overheating than soybeans, but controlled heat treatment of hay shows promise as a means of protecting the protein in a portion of the dietary forage.

Table 1. Effect of feeding steam heated hay on apparent digestibility[1] of dietary nutrients (Trials 1 and 2).[2]

	Trial 1			Trial 2				
Item	U	H	$P > F$[3]	C	SBM	U	H	$P > F$[3]
				(%)				
DM	61.2	53.8	< .001	62.2^{a}	62.7^{a}	62.6^{a}	60.6^{b}	.026
OM	ND	ND	. . .	63.8^{a}	64.6^{a}	64.2^{a}	62.3^{b}	.014
NDF	44.2	51.6	< .001	53.0^{a}	53.1^{a}	50.5^{b}	51.4^{ab}	.014
ADF	40.5	41.2	.584	44.5^{ab}	44.9^{a}	43.0^{b}	39.5^{c}	<.001
ADIN	-12.2	35.8	< .001	-27.0^{c}	-22.2^{bc}	-18.0^{b}	1.8^{a}	<.001
Total N	69.0	46.7	< .001	61.4^{b}	67.2^{a}	62.1^{b}	52.4^{c}	<.001

[a,b,c]Means with different superscripts in Trial 2 differ ($P < .05$).

[1] Apparent digestibility estimated using indigestible ADF as an internal marker.

[2] U = Unheated alfalfa hay; H = steam heated alfalfa hay; C = control diet; SBM = soybean meal; OM = organic matter; ND = not determined.

[3] Probability of a significant treatment effect.

Table 2. Effect of feeding steam heated hay on DMI, milk production, and concentrations of rumen metabolites (Trials 1 and 2).[1]

	Trial 1			Trial 2				
Item	U	H	$P > F$[2]	C	SBM	U	H	$P > F$[2]
DMI, kg / d	22.9	23.4	.318	23.4^{b}	24.2^{ab}	23.5^{b}	25.2^{a}	.003
Milk, kg / d	28.2	27.2	.002	33.4^{c}	35.1^{ab}	34.1^{bc}	35.7^{a}	.018
3.5% FCM, kg / d	26.7	26.5	.736	34.7	35.3	36.6	35.7	.469
Fat, %	3.17	3.34	.168	3.69	3.59	3.83	3.52	.284
Fat, kg / d	.90	.91	.707	1.22	1.26	1.31	1.25	.574
Protein, %	3.07	3.01	< .001	2.96	2.95	2.92	2.97	.626
Protein, kg / d	.87	.82	.001	.98^{b}	1.03^{ab}	.99^{b}	1.06^{a}	.053
Lactose, %	4.68	4.65	.234	4.84	4.85	4.85	4.87	.560
Lactose, kg / d	1.32	1.26	.001	1.61^{c}	1.70^{ab}	1.65^{bc}	1.74^{a}	.010
SNF, %	8.43	8.34	.013	8.44	8.44	8.41	8.49	.543
SNF, kg / d	2.38	2.27	<.001	2.81^{c}	2.96^{ab}	2.86^{bc}	3.02^{a}	.019
Rumen Metabolites								
pH	6.42	6.51	.605	6.43^{a}	6.58^{a}	6.42^{a}	6.10^{b}	.011
Ammonia, m*M*	18.0	9.2	.007	23.4^{ab}	28.0^{a}	22.6^{b}	13.3^{c}	.002
Acetate, mol %	68.6	69.4	.345	64.0	64.3	64.2	64.0	.537
Propionate, mol %	18.7	19.8	.513	18.9	18.5	18.3	19.4	.201
Butyrate, mol %	9.6	8.3	.014	11.3	11.1	11.5	10.8	.357
Isobutyrate, mol %	1.07	.50	.014	1.59^{b}	2.07^{a}	1.40^{b}	1.00^{c}	.032
"Isovalerate," mol %	1.39	.45	.008	2.09^{ab}	2.39^{a}	1.70^{bc}	1.50^{c}	.019

[a,b,c,d]Means with different superscripts in Trial 2 differ ($P < .05$).

[1] U = Unheated alfalfa hay; H = steam heated hay; C = control diet; SBM = soybean meal.

[2] Probability of a significant dietary treatment effect.

A NET CARBOHYDRATE AND PROTEIN SYSTEM FOR EVALUATING CATTLE DIETS: I. RUMINAL FERMENTATION

J.B. RUSSELL, J.D. O'CONNOR, D.G. FOX, P.J. VAN SOEST and C.J. SNIFFEN

Introduction

In ruminants, feedstuffs are fermented in the rumen prior to gastric and intestinal digestion, and this fermentation has confounded the prediction of animal performance from dietary ingredients. Over the years, there has been considerable improvement in the feeding of ruminants, but this progress has been based on an empirical approach which has largely treated the rumen as a "black box". The experience of other scientific disciplines has shown us that a mechanistic understanding is usually needed for sustained development. If ruminant nutrition is to progress past the point where diets are continually tested in virtually infinite combinations, the details of the fermentation must be considered.

Results and Discussion

The Cornell Net Carbohydrate and Protein System (CNCPS) has a kinetic submodel which predicts ruminal fermentation. The rumen microbial population is divided into bacteria which ferment structural carbohydrate (SC) and those that ferment non-structural carbohydrate (NSC). Protozoa are accommodated by a decrease in the theoretical maximum growth yield (.50 versus .40 g cells/g carbohydrate fermented), and the yields are adjusted for maintenance requirements (.05 versus .150 g cell dry weight/g carbohydrate fermented/h for SC and NSC bacteria, respectively). Bacterial yield is decreased when forage neutral detergent fiber (NDF) is less than 20% (2.5% for every 1% decrease in NDF). The SC bacteria utilize only ammonia as a nitrogen source, but the NSC bacteria can utilize either ammonia or peptides. The yield of NSC bacteria is enhanced by as much as 18.7% when proteins or peptides are available. The NSC bacteria produce less ammonia when the carbohydrate fermentation (growth) rate is rapid, but 34% of the ammonia production is insensitive to the rate of carbohydrate fermentation. Ammonia production rates are moderated by the rate of peptide and amino acid uptake (.07 g peptide/g cells/h), and peptides and amino acids can pass out of the rumen if the rate of proteolysis is faster than the rate of peptide utilization. The protein sparing effect of ionophores is accommodated by decreasing the rate of peptide uptake by 34%.

The literature contains an abundance of performance data, but few studies have 1) described the diets so that the rates of carbohydrate fermentation and protein degradation can be estimated with accuracy and 2) reported the flow of microbial protein and undegraded feed to the lower gut. Thus, only certain studies could be used to validate the model (Garrett et al., 1987; Glenn et al., 1989; Song and Kennelly, 1989; McCarthy et al., 1989; and Robinson and Sniffen, 1985). These studies used Holstein cows and steers which were fed corn, barley, and silage-based diets at various intakes. The regression coefficient of the observed and predicted bacterial nitrogen flow from the rumen was 0.94 ($R^2 = 0.88$) and the slope was 0.94.

Conclusions

We have used this submodel to design feeding experiments and evaluate the results. The model correctly predicted metabolizable protein (microbial plus undegraded feed) supply needed by growing calves. Similar results were observed in field studies with beef and dairy cattle. We are encouraged by these results and feel that the CNCPS can account for the effect of variations in feed carbohydrate and protein fractions on microbial yield, feed protein escaping ruminal degradation, and carbohydrate utilization in the rumen.

A NET CARBOHYDRATE AND PROTEIN SYSTEM FOR EVALUATING CATTLE DIETS: II. CARBOHYDRATE AND PROTEIN AVAILABILITY

C.J. SNIFFEN, J.D. O'CONNOR, P.J. VAN SOEST, D.G. FOX AND J.B. RUSSELL

Introduction

The Weende system for proximate analysis and the TDN system have been used for about a century as the basis for predicting the energy and protein available from feedstuffs. Net energy (NE) systems were developed to adjust for methane, urinary, and heat increment losses. The NE systems have worked well under standard feeding conditions, but the tabular value of NE for a particular feed is typically computed from TDN and represents the average expected value based on a group of feeds rather than the NE that will be derived by a particular group of cattle eating that feed. Because the feeding conditions of cattle are variable and often unique, accurate NE values usually are not available. Crude and digestible protein determinations do not completely account for the dynamics of ruminal fermentation and the potential loss of nitrogen as ammonia. The Cornell Net Carbohydrate and Protein System (CNCPS) has equations that estimate the fermentation and passage of feed carbohydrate and protein fractions. This information can be used as a basis for predicting ME and protein absorption.

Results and Discussion

In the CNCPS, structural carbohydrate (SC) and nonstructural carbohydrate (NSC) are estimated from the sequential NDF analyses of the feed. Data from the literature are used to predict fractional rates of SC and NSC degradation. Crude protein is partitioned into five fractions. Fraction A is NPN, which is trichloroacetic (TCA) acid-soluble N. Unavailable or protein bound to cell wall (Fraction C) is derived from acid detergent insoluble nitrogen (ADIP), and slowly degraded true protein (Fraction B3) is neutral detergent insoluble nitrogen (NDIP) minus Fraction C. Rapidly degraded true protein (Fraction B1) is TCA-precipitable protein from the buffer-soluble protein minus NPN. True protein with an intermediate degradation rate (Fraction B2) is the remaining N. Protein degradation rates are estimated by an in vitro procedure that uses *Streptomyces griseus* protease, and a curve-peeling technique is used to identify rates for each fraction. The amount of carbohydrate or N that is digested in the rumen is determined by the relative rates of degradation and passage. Ruminal passage rates are a function of DMI, particle size, bulk density, and the type of feed that is consumed (e.g., forage vs. cereal grain).

Conclusions

Validations indicate that the digestion and passage rates used are appropriate for typical mixed diets. However, we have found that users may need to adjust these rates, as well as the microbial growth rates, based on their knowledge of factors that may result in unusually low or high digestion and passage rates. The ruminal digestion rates assume a normal pH, and the submodel of the rumen adjusts microbial protein yield in high grain rations in which pH can be expected to be < 6.0 to 6.2. However, it does not automatically adjust microbial growth on the SC fraction which is very pH-sensitive. In evaluation of data from experiments with 0 to 10% forage, we concluded that the use of a growth rate of 0 for the SC bacteria was appropriate for this type of diet. The associative effects of N sources are accounted for primarily by determining the ammonia and peptide requirements of the SC and NSC pools. By adding a calculation to estimate the branched-chain VFA requirement for SC bacteria as described in the submodel of the rumen, the positive effects of using true protein to provide ammonia for SC bacteria in high-forage diets can be accounted for.

A NET CARBOHYDRATE AND PROTEIN SYSTEM FOR EVALUATING CATTLE DIETS: III. CATTLE REQUIREMENTS AND DIET ADEQUACY

D.G. FOX, C.J. SNIFFEN, J.D. O'CONNOR, J.B. RUSSELL and P.J. VAN SOEST

Introduction

National Research Council nutrient requirements are the standards most often used in the United States to predict requirements of cattle. These standards typically have been based on research data from uniform cattle with little environmental stress and do not account for all the variables of actual field conditions. Cattle can differ significantly in their biological potential, and these animals are produced in nearly every environmental condition. Feed energy and protein vary widely from farm to farm and depend on soil type, plant variety, growth condition, fertilization, harvest, and storage management.

Results and Discussion

The Cornell Net Carbohydrate and Protein System (CNCPS) has equations for predicting nutrient requirements, feed intake, and feed utilization over wide variations in cattle (frame size, body condition, and stage of growth), feed carbohydrate and protein fractions and their digestion and passage rates, and environmental conditions. Independent data were used to validate the ability of the CNCPS to predict responses compared to National Research Council (NRC) systems. With dry matter intake (DMI) in steers, the CNCPS had a 12% lower variation of the Y estimate and three percentage units less bias than the NRC system. For DMI in heifers, both systems had a similar variation, but the NRC had four percentage units less bias. With lactating dairy cows' DMI, the CNCPS had a 12% lower variation. Observed NE_m requirement averaged 5% under NRC and 6% under CNCPS predicted values at temperatures above 9°C but were 18% over NRC and 9% under CNCPS at temperatures under 9°C. Energy retained was predicted with an R^2 of .80 and .95 and a bias of 8 and 4% for the NRC and CNCPS, respectively. Protein retained was predicted with an R^2 of .75 and .85 with a bias of 0 and -1% for NRC and CNCPS, respectively. Biases due to frame size, implant, or NE_g were small. Body condition scores predicted body fat percentage in dairy cows with an R^2 of .93 and a variation of 2.35% body fat. The CNCPS predicted ADG allowed by metabolizable protein from the diet with a bias of 1.6% with a variation of .07 kg compared to values of -30% and .10 kg, respectively, for the NRC system.

The CNCPS accounts for the following relationships: 1) feed ME as a function of NDF, lignin, digestion and passage rates, 2) bacterial yield as a function of SC and NSC pools, the rate at which the carbohydrates and proteins are degraded, and ruminal pH, 3) ruminal N requirements in relation to microbial growth on intake of SC and NSC, 4) the influence of carbohydrate on ammonia production, 5) the ME cost to excrete excess N, 6) maintenance requirement sensitive to animal and environmental conditions, 7) growth requirements sensitive to variations in frame size and anabolic implants, 8) optimum growth rate for herd replacements, 9) body condition score and energy reserves.

Conclusions

The CNCPS provides a biologically meaningful structure for evaluating cattle diets under widely varying conditions. Validations have indicated that it gives realistic estimates of animal performance. Although certain parameters will need future refinement with lower aggregation models, the present model provides a structure that is biologically meaningful and can be used to develop cattle diets under diverse conditions.

ESTIMATING NET ENERGY AND ABSORBED PROTEIN REQUIREMENTS FOR INDIVIDUAL MILK COMPONENTS

R.G. DADO, D.R. MERTENS and G.E. SHOOK

Introduction

Current nutrient requirements for milk production of the NRC are based solely on milk fat concentration. Milk protein and lactose concentration are assumed to be correlated to milk fat or are assumed to be constant. Multiple component pricing of milk will serve to stimulate testing for milk components and make the assumptions built into the NRC recommendations unnecessary. Genetic selection and biotechnological advances offer the potential to change milk composition in response to consumer needs. Defining nutrient requirements for each milk component may improve the efficiency and profitability of milk production because the requirements for energy and protein for individual milk components vary greatly. The objective of this research was to develop absorbed protein and net energy requirements for milk fat, protein and lactose and compare them to current NRC recommendations.

Materials and Methods

Metabolic pathways describing the transformation of nutrients into each milk component were obtained from the literature. Precursors for milk components (acetate, propionate, b-hydroxybutyrate, palmitate, stearate, glycerol and amino acids) were traced from absorption into the blood to the synthesis of protein, fat and lactose in the mammary gland. Energy for synthesis was estimated assuming 20 kcal/mol of ATP. Amino acid analyses of milk protein were used to estimate the total absorbed protein (AP) requirement for protein synthesis.

Almost all glucose used for milk synthesis comes from gluconeogenesis. Propionate and glucogenic amino acids are the major precursors of glucose. Literature reports suggest that amino acids contribute 0 to 50% of the glucose used by ruminants. Sensitivity analysis in which amino acid contributions to glucose were varied from 0 to 50% were used to assess the magnitude of gluconeogenesis from amino acids.

Fat synthesis was modeled to have a fatty acid profile similar to milk fat. Most long chain fatty acids were assumed to be absorbed by the mammary gland preformed. These preformed fatty acids originated from the diet (50%) and adipose tissue (50%). Acetate was the precursor for short-chained fatty acid synthesis in the mammary gland and long-chained fatty acid synthesis in adipose tissue.

Standard milk production and composition were based on the analysis of 50,769 monthly DHI records from 575 cows in 63 herds in Wisconsin (Kalscheur, Combs and Shook, unpublished). This summary indicated an average milk production (± standard deviation) of 25.1 (± 7.8) kg/day containing 3.71 (± .64) percent fat, 3.27 (± .34) percent protein and 5.08 (± .31) percent lactose. These data were used to provide ranges for evaluating differences between our proposed requirements and those of the NRC.

Results and Discussion

The efficiency of converting available N (absorbed N-tissue balance N-maintenance N) to milk was used to evaluate the sensitivity of amino acid use for gluconeogenesis. When amino acids supplied 15% of the glucose, the efficiency of remaining amino acids for milk protein synthesis exceeded 100%. This suggests that amino acids produce a maximum of

15% of the glucose in moderate to high producing dairy cows. The efficiency of use of remaining amino acids for milk protein synthesis after gluconeogenesis is approximately 90% when 10% of the amino acids are used to synthesize glucose. The use of amino acids for gluconeogenesis indicates that non-amino acid containing milk components, such as fat and lactose, have an AP requirement for glucose production (Table 1).

Net energies of milk components were related to the heats of combustion of individual milk components (Table 1). Theoretical metabolizable energy (ME) requirements based on ATP required for synthetic pathways underestimated the total ME required according to NRC and were increased for each milk component based on marginal changes in ME requirements for milks of differing composition reported by the NRC (Table 1).

There were few differences between the NRC requirements and those we proposed for cows with milk compositions assumed by the NRC (Table 2). However, substantial differences occur when milk is produced that is high or low in fat or protein. These differences will result in substantial changes in ration composition for cows producing milk in excess of 25 kg/day.

Conclusions

Increased analysis of milk for protein and lactose content, as well as for fat, provides the opportunity to formulate rations that more efficiently meet the needs of dairy cows provided requirements are available for individual milk components. A system of absorbed protein and net energy requirements has been developed based on biological and chemical principles that can serve as the basis for formulating rations to meet specific milk component yields.

Table 1. Absorbed protein (AP), net energy of lactation (NEL) and metabolizable energy (ME) requirements for milk components assuming that 10% of the glucose comes from gluconeogenesis of amino acids.

Milk component	AP	NEL	Theoretical ME	Adjusted ME
	kg/kg component	-------Mcal/kg component		-------
Fat	.127	9.23	11.40	13.43
Protein	1.069	5.71	6.43	7.57
Lactose	.136	3.95	5.11	6.02
Milk volume				.144

Table 2. Difference in nutrient requirements between the proposed system based on requirements for milk components and the NRC.

Description	Milk composition			AP		NEL		ME	
	Fat	Protein	Lactose	Proposed	NRC	Proposed	NRC	Proposed	NRC
	------	%	------	kg/kg component		--------	Mcal/kg	--------	
NRC, medium fat	3.5	3.3	5.0	0.047	0.047	0.71	0.69	1.15	1.15
Average	3.7	3.3	5.1	0.047	0.048	0.73	0.71	1.18	1.19
Avg fat, low protein	3.7	2.9	4.7	0.042	0.048	0.69	0.71	1.13	1.19
Avg fat, high protein	3.7	3.6	5.4	0.051	0.048	0.76	0.71	1.22	1.19
Low fat, high protein	3.1	3.6	5.4	0.050	0.045	0.70	0.65	1.14	1.09
High fat, low protein	4.4	2.9	4.7	0.043	0.052	0.76	0.77	1.22	1.30
NRC, high fat	4.5	3.7	5.0	0.052	0.053	0.82	0.78	1.31	1.32

DEVELOPING A SYSTEM TO BALANCE RATIONS FOR ABSORBED PROTEIN AND TO ESTIMATE FEED COSTS FOR MILK COMPONENTS

D.R. MERTENS, R.G. DADO and G.E. SHOOK

Introduction

Requirements for absorbed protein (AP) and net energy (NEL) for the production of milk fat, protein and lactose have been derived from metabolic pathways of component synthesis and their heats of combustion. Formulating rations to meet these requirements necessitates that feed nutritive values be expressed in the same units. Although the combustible energy in milk components can be directly related to the NEL value of feeds, AP protein values are not as easily defined because protein undergoes numerous transformations in the rumen and lower digestive tract. The system published by the NRC can be used to evaluate rations for AP after they are formulated, but cannot be used to directly determine the amounts of feed ingredients needed to meet a given AP requirement. The objective of this research was to develop a system of equations for formulating rations for the NEL and AP requirements of milk components and assess the relative feed costs of producing milk fat, protein and fat.

Methods

The nutritive requirements for milk components were those developed by Dado et al. (1993). Quantitative relationships among nitrogen transformations were taken from the NRC. According to the NRC, microbial protein can be limited by either ruminally available protein or ruminally available energy. Compared to balancing rations for crude protein, formulating rations for AP requires two equations, one when energy is limiting and another when protein is limiting bacterial crude protein synthesis (BCP). The ration formulation that meets the minimum of these two equations is the only one resulting in adequate AP for meeting animal requirements. The protein system of the NRC was used to obtain AP values when limited by energy (APE) or when limited by protein (APP) for each feed (Table 1). When combined with NEL requirements and feed values, a system of three equations can be used to meet the AP and NEL requirements (APR, NELR) for each milk component. Corn and 44% soybean meal were selected as reference energy and protein supplements, respectively, because they are commonly used to formulate dairy rations, have widespread availability, and have relatively stable long-term prices.

Results and Discussion

The 2 x 3 matrix (Table 2) can be solved using linear programming (LP) methods, but LP methods require an objective function such as cost to be minimized. To derive a system that is more easily solved by simultaneous equations requires that the matrix be 2 X 2, i.e., two feeds and two requirements. This can be accomplished by using the appropriate combination of NEL and APE or NEL and APP for each milk component. In the first instance, it is explicitly assumed that energy limits BCP, while in the latter instance protein is assumed to limit BCP. When these two sets of 2 X 2 matrices are solved to equal solutions, the NELR:APR ratio that optimizes BCP and AP can be determined. For corn and soybean meal, this ratio is 12.36. When NELR:APR = 12.36, both NEL and AP requirements are met exactly and all ruminally available protein is trapped by bacteria.

When the NELR:APR ratio is less than 12.36, ruminally available energy limits BCP and the NEL and APE matrix is used. Conversely, when the NELR:APR ratio is more than 12:36, ruminally available protein limits BCP and the NEL and APP matrix is used. The marginal feed requirements and costs for single milk components can be calculated assuming the base ration was formulated to meet the base requirements for the other milk components and their production was not changed. The NELR:APR ratio for fat (72.68) and lactose (29.04) exceed 12.36, requiring the use of the NELR and APP matrix; whereas the ratio for milk protein (5.34) is less than 12.36 and requires the use of the NEL and APE matrix. The marginal feed requirements and costs for 1 kg of each milk component is given in Table 3. The use of the new system of requirements indicates that the feed cost for producing a kg of milk protein is substantially higher than that for producing milk fat or lactose.

Conclusion

A system of simultaneous equations can be developed and solved that derive the quantities of corn and soybean meal needed to meet the NEL and AP requirements for individual milk components. These equations require the intermediate calculations of AP yield from each feed depending upon the limit on ruminal bacterial protein synthesis, protein or energy. Based on newly derived nutrient requirements and a system for balancing rations based on AP, the feed cost for producing milk protein was found to be substantially higher than the feed costs for producing milk fat or lactose.

Table 1. Calculation of absorbed protein when energy is limiting bacterial protein synthesis (APE) and of absorbed protein when protein is limiting bacterial protein synthesis (APP) for use in the ration formulation matrix in Table 2.

Feed component or matrix coefficient	Corn	Soybean meal
CP[a] (kg/kg)	0.100	0.499
DUP[b] (kg/kg)	0.042	0.140
NEL[c] (Mcal/kg)	1.96	1.94
BCP from NEL[d] (kg/kg)	0.140	0.139
DBP from NEL[e] (kg/kg)	0.090	0.089
APE[f] (kg/kg)	0.131	0.229
RAP[g] (kg/kg)	0.063	0.399
BCP from RAP[h] (kg/kg)	0.057	0.359
DBP from RAP[i] (kg/kg)	0.036	0.230
APP[j] (kg/kg)	0.078	0.370

[a] Crude protein (NRC, 1989).

[b] Digestible undegraded protein = .80*.

[c] Net energy of lactation (NRC, 1989) UNDEG*CP.

[d] Bacterial crude protein synthesized when energy is limiting (BCPE) = NEL*6.25*.01145.

[e] Digestible bacterial true protein when energy is limiting (DBPE) = .80*.80*BCPE.

[f] Absorbed protein when energy limits bacterial growth = DBPE + DUP.

[g] Ruminally available protein = CP*(DEG+ RECYCLED)/100.

[h] Bacterial crude protein synthesized when protein is limiting (BCPP) = .90*RAP.

[i] Digestible bacterial true protein when protein is limiting (DBPP) = .80*.80*BCPP.

[j] Absorbed protein when protein limits bacterial growth = DBPP + DUP.

Table 2. Simplified equation matrix for determining the amounts of corn and soybean meal needed to meet absorbed protein (APR) and net energy requirements (NELR) for milk components.

Row description	Corn	Soybean meal	= RHS
NEL	1.96	1.94	> = NELR
APE[a]	.131	.229	> = APR+ .124
APP[b]	.078	.370	> = APR

[a] Absorbed protein when energy limits bacterial growth.

[b] Absorbed protein when protein limits bacterial growth.

Table 3. Amount of feed required and feed costs for 1 kg of milk component when each is produced independently of the others.

Milk component	Ground corn	44% Soybean meal	Feed cost[a]
	(kg)	(kg)	$
Fat	5.52	-.82	.49
Lactose	2.09	-.07	.25
Protein	-3.94	6.92	1.41

[a] Ground corn = $.129/kg DM and 44% soybean meal = $.276/kg DM

U.S. DAIRY FORAGE RESEARCH CENTER ANNUAL DAIRY OPERATIONS REPORT, JANUARY 1993

L.L. STROZINSKI - HERD MANAGER

The research center herd count stands at 570 (284 cows and 286 replacement heifers). We are currently milking 230 cows which are yielding an average of 65.5 pounds of milk per day. Our current DHIA rolling herd average stands at 18,390 pounds of milk, 642 pounds of fat and 569 pounds of protein. During the past year our average milk production peaked at 18,600 pounds, then dropped to 18,065 pounds and is now again increasing. In 1992 the farm marketed 5.1 million pounds of milk to provide total milk receipts of $659,000. Sales of cull cows and bull calves brought in an additional $83,430. Reproductive performance of the herd is satisfactory. Average days open for the herd is currently 112 days with a projected calving interval of 12.7 months. Our heifers continue to enter the milking herd at an average weight of 1200 pounds and calve at an average age of 24 months. First calf heifers currently make up 33% of the milking herd. Average age of the milking herd is 49 months. The registered herd was classified by the Holstein Association in April of 1992 with the following results: 19 very good, 90 good plus, 55 fair and 13 poor. The dairy forage herd breed age average stands at 97.9%. Ninety five percent of the herd is registered.

Research use of the herd continues to be high throughout the year. There has been a trend toward longer term studies with larger numbers of animals. This trend has helped to streamline our operation and utilize labor more efficiently. Currently 46% of the milking herd is on trial. During the year, 294 animal units were used on 12 research trials. In addition, 230 cows were part of a cooperating study with the University of Wisconsin School of Veterinary Medicine. Focus of this study was milk fever and its prevention.

Several modifications have been made to the livestock facility during the past year. The entire lighting system has been modified to a more energy efficient system. Sodium bulbs have been installed in selected areas and all fluorescent fixtures have been modified to utilize high efficiency ballasts and bulbs. Tunnel ventilation has been installed in both tie stall barns to provide an improved, energy efficient air flow during the warm season. Continued efforts are under way to scrutinize energy consumption at the farm and maximize energy efficiency wherever possible. A new feed wagon has been purchased and will be used to streamline our TMR feeding program. A new cattle weighing system has also been purchased which will link with the current computerized milking parlor system. The system will automatically identify and weigh cows as they exit the milking parlor and transmit the data directly to the office computer.

An increased emphasis was placed on pasture management and utilization at the farm this past summer. The large pasture immediately north of the main facility was divided into paddocks and grazed rotationally by the young stock and dry cows. Despite a very dry early summer, satisfactory animal performance was maintained throughout the entire summer with no supplemental feeding until autumn. In most previous years, supplemental feeding was needed by late July. In addition to pasturing benefits, several paddocks were harvested for hay. Continued emphasis will be placed on pasture utilization and intensive grazing in the summer of 1993.

A major change was made in the labor schedule in the dairy unit this year. Basically the change centered around increasing the amount of time that the milking parlor is operated by one person. This change increased milking parlor efficiency in terms of cows milked per man per hour. Labor saved was redirected into the unit.

The dairy operation continues to benefit from the efforts of not only an excellent dairy crew but also the cooperation and assistance of the farm crew as well as the maintenance and repair personnel. The performance and extra efforts of all these individuals are greatly appreciated.

U.S. DAIRY FORAGE RESEARCH CENTER ANNUAL FIELD OPERATIONS REPORT, JANUARY 1993

R.P. WALGENBACH

Richard P. Walgenbach

The winter of 1991/92 killed out about 40 acres of winter wheat, severely damaged about 75 acres of alfalfa and negatively impacted most alfalfa fields. In general, winter damage to wheat and alfalfa was very severe in the southern half of Wisconsin. Frost heaving of alfalfa plants in late winter and early spring also was prevalent this past season. The growing season consisted of a cold wet spring, a droughty early summer, a frost on Father's Day (21 June), below normal summer temperatures, and an almost sunless and very wet autumn.

These conditions caused much anxiety for state farmers this year. Considering the weather of the past growing season, overall crop production at the USDFRC farm did quite well. The winter injury and early season drought reduced alfalfa yields but produced excellent quality forage. It also affected early season growth of newly seeded alfalfa. Corn growth did not appear to be affected by this drought period. All but about 3 acres of corn escaped the Father's Day frost. Soybeans emerged poorly in both tilled and no-tilled acres. This soybean emergence problem was most likely due to surface compaction and lack of moisture at critical times. Weed control in all crops offered a challenge this past season. Timely cultivation and applications of post emergence chemicals kept weed competition to a reasonable level in most fields.

About 60 acres of soybeans were drilled into standing corn stalks with a Lilliston no-till drill this second season of soybean production. Corn also was no-till planted into 39 acres of autumn killed alfalfa. These cropping practices produced excellent results this past season and will be continued and expanded to additional acres in 1993. Attempts to plant corn no-till into manured soybean and corn residues were frustrated by residues of manure keeping soils wet and cool, surface compaction from spring applied manure, and using a planter not adequately set-up for no-till planting. We are exploring approaches to deal with the problems associated with no-till and reduced-till planting on livestock farms.

The chemical storage and handling facility was completed near the end of the growing season and is currently in use. The costs associated with the lease of equipment at the farm was evaluated and a decision was made to purchase our leased tractors. The John Deere 4760 (170 h.p.) and 4255 (120 h.p.) were purchased at the end of FY 92 and the beginning of FY 93, respectively. Two additional tractors (85 h.p.) will be purchased as funds allow. We also initiated an attempt to replace the manned guard at gate 8 with an unmanned electronic gate. This was not completed in FY 92 because of an underestimate of the cost of the project. This project has been resubmitted and is currently being advertised for bids. We also have received notification that funding is being provided to replace our clay lined lagoon with a more permanent structure. Plans for expansion of the conference room and employees lunch room have been drawn up and are waiting for funding.

All underground fuel and oil storage tanks have been removed and replaced with new double walled tanks. The tanks are also monitored continuously to detect leaks. An area of contamination accrued under the fuel tanks. This soil was removed and is currently stored under a plastic cover. The plans are to remediate this soil by mixing it with manure and allowing it to heat. This process is currently waiting for approval from the Wisconsin Department of Natural Resources.

The field operations continue to enjoy excellent effort from the field and maintenance staff as well as cooperation from the barn crew. The Center's management and research staff are fortunate to have this group of knowledgeable, cooperative, hardworking and congenial people to work with.

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